Peter H. Wiernik
Janice P. Dutcher
Morie A. Gertz
Editors

Neoplastic Diseases of the Blood

Sixth Edition

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The editors of the sixth edition of this book dedicate it to our families; to our mentors; to the hundreds of fellows we have trained over the years, many of whom have gone on to lead major cancer research and treatment programs on several continents; and to the thousands of patients we have had the privilege to care for and learn from over the decades. We also honor the continuing influence on this edition by former editors of previous editions, Drs. George P. Canellos, John M, Goldman, Robert A, Kyle and Charles A. Schiffer.

Preface

The sixth edition of *Neoplastic Diseases of the Blood* is long overdue despite the fact that the fifth edition was published only five years ago, due to the fact that major progress in our understanding of the nature of hematologic malignancies and their treatment has occurred in the interim. This edition is current and up to date, drawing heavily on recent references, and is designed to be a readable, encyclopedic resource for established hematologists and oncologists as well as for trainees in our disciplines.

The chapter structure of the book follows essentially that of the fifth edition, with the addition of some new chapters in the myeloma section. This edition is also divided into five sections like previous editions, each developed and managed by an editor: Chronic Leukemias and Related Disorders (Peter H. Wiernik), Acute Leukemias (Peter H. Wiernik), Myeloma and Related Disorders (Morrie Gertz), Lymphomas (Peter H. Wiernik), and Supportive Care (Janice P. Dutcher). Over 100 authors, many new to this edition, have contributed their expertise to this work.

Our sincere hope is that patients with hematologic malignancies will directly benefit from our work. This hope drove us to take on and complete this huge task that is the creation of this book.

We thank the publisher, Springer Medicine, for invaluable assistance during all phases of the development of the book. Special thanks to Maureen Alexander, Developmental Editor at Springer, for her highly professional continual interactions with the editors and all authors that was instrumental in bringing this project to a close in a timely manner, and to Andy Kwan, Editor, Clinical Medicine for Springer, for overseeing the project from beginning to end.

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Contents

Part I Chronic Leukemias and Related Disorders

1 A History of the Chronic Leukemias. 3
George P. Canellos and Matthew S. Davids

2 Epidemiology and Etiology of Chronic Myeloid Leukemia 9
Maren Rohrbacher and Joerg Hasford

3 Pathology of the Chronic Myeloid Leukemias 19
Barbara J. Bain

4 Molecular Biology and Cytogenetics of Chronic Myeloid Leukemia. 29
Marina Konopleva, Alfonso Quintás Cardama, Hagop Kantarjian, and Jorge Cortes

5 Diagnosis and Treatment of Chronic Myeloid Leukemia 49
Charles A. Schiffer

6 Etiology and Epidemiology of Chronic Lymphocytic Leukemia 69
Helen E. Speedy, Daniel Catovsky, and Richard S. Houlston

7 Morphology and Immunophenotype of Chronic Lymphocytic Leukemia 79
Mir Basharath Alikhan and Girish Venkataraman

8 The Genomic and Epigenomic Landscape of Chronic Lymphocytic Leukemia. 99
Jonathan C. Strefford, Renata Walewska, and David G. Oscier

9 Treatment of Chronic Lymphocytic Leukemia and Related Disorders. 117
Deepa Jeyakumar and Susan O'Brien

10 Hairy Cell Leukemia. 135
Sonia Ali and Alan Saven

Part II Acute Leukemias

11 History of Acute Leukemia 155
Emil J Freireich

12 The Etiology of Acute Leukemia 161
J. N. Nichol, M. Kinal, and W. H. Miller Jr.

13 Epidemiology and Hereditary Aspects of Acute Leukemia 179
Logan G. Spector, Erin L. Marcotte, Rebecca Kehm, and Jenny N. Poynter

14 Classification of the Acute Leukemias: Cytochemical and Morphologic Considerations 197
N. Nukhet Tuzuner and John M. Bennett

15 Immunobiology of Acute Leukemia 237
Elisabeth Paietta

16 Cytogenetics of Acute Leukemia 281
Nyla A. Heerema and Susana Catalina Raimondi

17 Diagnosis and Treatment of Childhood Acute Lymphoblastic Leukemia 307
Melinda Pauly and Lewis B. Silverman

18 Diagnosis and Treatment of Adult Acute Lymphoblastic Leukemia 337
Nicola Gökbuget and Dieter Hoelzer

19 Diagnosis and Treatment of Acute Myeloid Leukemia in Children. 359
Brenton G. Mar and Barbara A. Degar

20 Diagnosis and Treatment of Adult Acute Myeloid Leukemia Other than Acute Promyelocytic Leukemia 375
Peter H. Wiernik

21 Acute Promyelocytic Leukemia 409
Peter H. Wiernik, Robert E. Gallagher, and Martin S. Tallman

22 Therapy-Related Acute Myelogenous Leukemia. 465
Hyung Chan Suh and H. Phillip Koeffler

23 The Myelodysplastic Syndromes 483
Kenneth Miller and Monika Pilichowska

Part III Myeloma and Related Diseases

24 History of Multiple Myeloma. 511
David P. Steensma and Robert A. Kyle

25 Monoclonal Gammopathy of Undetermined Significance 525
Malin Hultcrantz and Ola Landgren

26 Smoldering Multiple Myeloma 531
María-Victoria Mateos and Jesús F. San-Miguel

27 Frail Patients with Newly Diagnosed Multiple Myeloma 539
Alessandra Larocca and Antonio Palumbo

28 Newly Diagnosed Multiple Myeloma in Transplant-Eligible Patients 551
Rajshekhar Chakraborty and Morie A. Gertz

29 Role of Hematopoietic Cell Transplantation for Myeloma 573
Heather Landau and Sergio Giralt

30 Solitary Plasmacytomas and Soft-Tissue Involvement in Multiple Myeloma. 585
Joan Bladé and Laura Rosiñol

31 Supportive Care in Multiple Myeloma 595
Simit Mahesh Doshi, Tom T. Noff, and G. David Roodman

32 POEMS Syndrome 609
Angela Dispenzieri

33 Waldenstrom's Macroglobulinemia 617
Steven P. Treon, Giampaolo Merlini, and Meletios Dimopoulos

34 Plasma Cell Leukemia 639
Nisha S. Joseph and Sagar Lonial

35 Prognosis of Myeloma/Genetics of Myeloma 645
Sébastien Robiou-Du-Pont, Jill Corre, and Hervé Avet-Loiseau

36 Immunoglobulin Light Chain Amyloidosis (AL) 651
Morie A. Gertz, Francis K. Buadi, Taimur Sher, and Angela Dispenzieri

Part IV Lymphoma

37 Historical Landmarks in an Understanding of the Lymphomas 675
Marshall A. Lichtman

38 Genetics in Lymphomagenesis 723
James R. Cerhan, Esteban Braggio, Susan L. Slager, and Anne J. Novak

39 Epidemiology and Hereditary Aspects of Hodgkin and Non-Hodgkin Lymphomas 755
Seymour Grufferman

40 Pathology of Non-Hodgkin and Hodgkin Lymphomas 773
Mariko Yabe and L. Jeffrey Medeiros

41 Immunology of the Lymphomas 827
Christopher Sequeira and Howard Ozer

42 Cytogenetics of Lymphomas 853
Meaghan Wall and Lynda J. Campbell

43 Diagnosis and Treatment of Hodgkin Lymphoma 903
Peter H. Wiernik

44 Radiotherapeutic Management of Lymphomas 939
John P. Plastaras and Eli Glatstein

45 Diagnosis and Treatment of Non-Hodgkin's Lymphoma of Adults 951
David G. Crockett, James O. Armitage, and Julie M. Vose

46 Non-Hodgkin Lymphoma of Childhood 975
Tony H. Truong, Sarah Alexander, and Sheila Weitzman

47 Lymphoma in Other Diseases 999
Jennifer Crombie and Dan L. Longo

Part V Supportive Care

48 Supportive Care for Patients with Leukemia: A Historical Perspective 1039
Charles A. Schiffer

49 Prevention of Infections in Patients with Hematological Malignancies 1047
Marcio Nucci and Elias J. Anaissie

50 Evaluation and Management of Bacterial and Fungal Infections in Patients with a Hematological Malignancy: A 2018 Update 1063
Maria Pia Franco, Jaime S. Green, and Jo-Anne H. Young

51 Viral Infections in Patients with Hematological Malignancies 1079
Jack W. Hsu, John W. Hiemenz, John R. Wingard, and Helen Leather

52 Therapeutic Cytapheresis, Plasmapheresis, and Plasma Exchange in Neoplastic Diseases of the Blood 1129
Janice P. Dutcher

53 Red Cell Transfusions in Patients with Hematologic Malignancies 1139
Karen E. King and Paul M. Ness

54 Platelet and Granulocyte Transfusion 1153
Janice P. Dutcher

55 Alternative Sources of Hematopoietic Stem Cells and Their Clinical Applications 1179
Filippo Milano, Shelly Heimfeld, and H. Joachim Deeg

56 HLA Typing in Support of Hematopoietic Cell Transplantation from Unrelated Donors 1193
Effie W. Petersdorf, Claudio Anasetti, Paul J. Martin, and John A. Hansen

57 Special Care of Blood and Marrow Hematopoietic Cell Transplant Recipient 1211
Syed A. Abutalib and Hillard M. Lazarus

58 Hematopoietic Growth Factors in the Supportive Care and Treatment of Patients with Hematologic Neoplasms 1247
Ryan W. Jacobs, Omotayo Fasan, Edward A. Copelan, and Belinda R. Avalos

59 Bleeding and Thrombosis in Hematologic Neoplasia 1263
Joseph J. Shatzel, Robyn Scherber, and Thomas G. DeLoughery

60 Psychological Aspects of Hematological Neoplasms 1291
Tomer Levin T. Levin and Judith Cukor

Index 1307

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Part I

Chronic Leukemias and Related Disorders

1 A History of the Chronic Leukemias

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Introduction

Leukemias, both chronic and acute, were not described separately. Historically, the state of excessive quantities of blood cells and secondary organ involvement is attributed to Velpeau in 1827 [1]; although initial observations suggested that the excess of corpuscles in the blood was related to suppuration, it appeared that those cases were more likely to be what is now known as chronic leukemia because of the duration of disease and systemic enlargement noted [2]. Virchow in 1845 introduced the term of white blood of "leukaemie." He accumulated nine cases and doubted that infection was an explanation for the process [3]. The first reported case in America was in 1852. Virchow also differentiated the leukemias according to "splenic" or "lymphatic" leukemia [4]. By 1870, Neumann proposed that splenic leukemias were derived from cells originating in the bone marrow [5]. Further separation of acute versus chronic leukemias was made by Ebstein (1889) introducing the term "acute leukemia," a disease with a very short survival [6]. It remained for Ehrlich to begin staining cells to separate granulocytes from lymphoid cells. This provided a test to classify leukemia even further than prior unstained descriptions [7]. The primitive cells, known as blasts, were further clarified by Naegeli in 1900 separating to some extent myeloblastic from lymphoblasts [8]. However, Turk in 1903 assembled all the "lymphoid" diseases under one classification known as lymphomatoses [9]. However, the true neoplastic nature of these disorders was confirmed when they could be induced in experimental animals by toxic chemical injection.

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Chronic Myeloid Leukemia

Chronic myeloid leukemia remained as defined by early investigations until 1960 when Nowell and Hungerford using the new technology of cytogenetics described a marker chromosome with deletion of genetic material known as the Philadelphia chromosome. This provided a test which defined CML as a clonal disorder [10]. This was further supported by studying patients who were heterozygous for isochromosomes of glucose-6-phosphate dehydrogenase which further defined the clonal origins of a number of other disorders [11]. In the 1970s, a greater understanding of the Philadelphia chromosome evolved. It was demonstrated by DeKlein that the human analog of the murine v-abl oncogene was translocated from its normal location on chromosome 9 to chromosome 22 and soon after that the reciprocal translocation of genetic material from chromosome 22 to chromosome 9 was described, resulting in a fusion gene BCR-ABL on chromosome 22 [12]. Two molecular rearrangements on BCR-ABL are possible but both result in a hybrid messenger RNA and thus a protein kinase [13, p. 210]. Modern molecular technology now allows for reassessment of minute quantities of the gene and facilitates the assessment of new therapeutics in assessing remission of the disease. A number of other features of CML have been attributed to the gene including proliferation, less apoptosis, and diminished cellular adhesion. The early therapeutic history of CML probably began with the use of arsenic trioxide (1% solution known as Fowler's solution). It was used on occasion in the nineteenth century with some transient benefit until the discovery of X-rays which were used to radiate the enlarged spleens. This resulted in significant improvements in signs and symptoms as well as blood counts [14]. The modern chemotherapy era for CML began with the demonstrations of the cytotoxic effects of nitrogen mustard [15]. An oral derivative of alkylating agent research was busulfan, which was introduced in 1953 and was, for a considerable period, the standard oral therapy of CML [16]. Other agents, such as chlorambucil and thiopurines as well as colchicine

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derivatives, were used. Because of the unpredictable and sometimes extensive bone marrow suppression with busulfan, another antimetabolite, hydroxyurea, was introduced and shown to be active [17]. The blastic phase of CML was quite refractory to effective treatment regimens used for acute myeloid leukemia and almost never resulted in a remission. Transient, but complete, remissions were however achieved when the blastic phase assumed lymphoid characteristics [18]. Vincristine and prednisone could produce remissions lasting months. The lymphoid nature of the blastic cells was confirmed by immunologic and cytologic tests [19]. Treatment also included interferon for a period before the introduction of the specific tyrosine kinase inhibitors directed at the BCR-ABL protein tyrosine kinase [20]. There was an initial success in producing hematologic as well as molecular remissions, and resurrecting the suppressed normal hemopoietic elements resulting in prolonged remissions [21]. The initial agent was imatinib but new mutations resulted in disease relapse and other more potent ABL inhibitors such as nilotinib, dasatinib, and ponatinib were introduced [22, 23]. The actions of these agents have dramatically altered the natural history and resulted in prolongation of survival obviating the need for allogeneic transplantation which was also a component of the treatment plan for some prior to the introduction of the tyrosine kinase inhibitors. Allotransplantation in its time was potentially curative, providing that there was a compatible donor, tolerance to graft-versus-host disease, and a survival from septicemia. Nonetheless, this could be curative and basically a manifestation of effective immunotherapy by allogeneic T cells against leukemic stem cells. Few patients are now candidates for this treatment as the new kinase inhibitors have had an overwhelming effect on the natural history of CML. It remains an effective therapy for those intolerant to all tyrosine kinase inhibitors (TKI) or with mutations conferring resistance to effect TKI therapy [24]. Allogeneic transplants were successful in the past when performed in the chronic phase with 12-year survival of 60–70%. The introduction of tyrosine kinase inhibitors, such as imatinib, has resulted in 8-year survivals of over 90% when used in the chronic phase. However, although uncommon, point mutations can occur and confer resistance to at least generations of inhibitors. The gatekeeper mutation, T3151, is the offender, but even for this mutation a recent TKI, ponatinib, has been introduced.

Chronic Lymphocytic Leukemia

The early histories of treating CML and CLL have much in common due to the limited availability of treatment options and the inability to distinguish the diseases with any degree of accuracy. In the 1940s and 1950s, Osgood [25–27] tested the hypothesis that whole-body external irradiation or administration of radioactive phosphorus could be titrated to control the leukocyte count at a level below 30×10^9/L. He claimed that this strategy was effective in patients with slowly progressing disease and that it could increase the chance of survival to 20 years. However, his results were not confirmed in later randomized trials comparing irradiation with chlorambucil and other alkylating agents [28, 29].

Progress in the clinical management of patients with CML has relied on improved understanding of the different types of disease and on improved prognosis. In the past, diseases diagnosed as CLL would have included a mixture of T- and B-cell leukemias, hairy cell leukemia, and a variety of other conditions associated with lymphocytosis. In contrast, the cells can now be identified accurately by cellular morphology, immunophenotype, cytogenetics, and other features [30] so that subtypes of disease can be grouped together and informative clinical trials can be designed.

It has been recognized for many years that cases of CLL have variable clinical courses [31]. The wide range of survival times for patient with CLL, from a few years to more than a decade, made treatment decisions difficult, particularly because some patients remained well even if they were not treated. This led to the development of staging systems, based on prognostic indicators and other criteria, to facilitate the choice of therapy for individual patients. The long list of prognostic indicators in CLL now includes age, sex, lymphocyte doubling time, cell morphology, and cytogenetic abnormalities [30]. In particular, patients with del(17p), del(11q), or complex cytogenetics have a poorer overall survival, whereas those with del(13q), trisomy 12, or normal cytogenetics have a better prognosis [32]. Patients whose CLL cells are positive for ZAP-70 tend to have a shorter time to treatment [33]. Testing for the mutational status of the immunoglobulin heavy-chain variable region (*IGHV*) also has significant prognostic impact, as patients with <2% similarity to germline DNA ("mutated") have a more indolent course and longer survival compared to those with >2% ("unmutated") [34, 35]. Recently, the presence of somatic mutations such as *TP53, NOTCH1, SF3B1*, and others has allowed further subgrouping of CLL [36, 37].

With regard to CLL therapy, most patients can be observed for a prolonged period of time after diagnosis without the need for treatment. This has been studied in several randomized clinical trials of early intervention with chemotherapy versus delaying therapy until patients meet treatment indications [38]. These indications as per the 2008 IW-CLL criteria include progressive cytopenias due to bone marrow infiltration by CLL, progressive organ-threatening, bulky lymphadenopathy, or progressive constitutional symptoms in the setting of progressive disease [39]. In the absence of these signs or symptoms, an overall survival advantage to early intervention with chemotherapy has never been demonstrated.

Once patients do meet treatment indications, numerous therapeutic options are available. Long-term low-dose treatment with the oral alkylating agent chlorambucil was a mainstay of CLL therapy for decades. For patients with more indolent forms of CLL, this approach regulated the size of the malignant B-cell clone without inducing major cytopenias or other toxicities [40]. The purine analog fludarabine was subsequently found to induce a higher rate of complete remission and a longer progression-free survival than chlorambucil, but as monotherapy it did not confer an overall survival advantage in a randomized trial [41]. Fludarabine plus the intravenous alkylating agent cyclophosphamide (FC) became a new standard of care when it was shown to induce deeper and more durable remissions than fludarabine alone [42]. In the 2000s, rituximab was added to FC to make the FCR regimen, which was pioneered by Keating and colleagues at MD Anderson Cancer Center [43, 44]. The promising results of their initial studies were subsequently confirmed by the German CLL Study Group in their CLL8 trial, which showed an overall survival advantage to FCR over FC as initial therapy for younger, fit patients with CLL [45]. This trial is notable for being the first randomized trial to demonstrate an overall survival benefit in CLL. More recently, a regimen comprised of the alkylating agent bendamustine with rituximab (BR) has become popular for frontline CLL therapy due to its high level of efficacy and generally favorable tolerability [46]. CLL10 is a large, randomized phase III trial that randomized previously untreated patients to FCR vs. BR [47]. The results demonstrated that FCR induced a higher rate of complete remission, more minimal residual disease (MRD) negativity, and a longer PFS than BR. However, an overall survival benefit has not yet emerged, and BR also provided excellent efficacy and tolerability. In particular, older, frailer patients had less severe cytopenias and infectious complications with BR compared to FCR, and therefore BR can be considered a standard regimen in this population. In patients under 65, FCR provided deeper and more durable remissions with relatively comparable toxicity, making it still the standard of care for this group. Recently published data by both the MD Anderson and German CLL groups on the long-term results of FCR treatment suggest that a substantial number of patients with mutated IGHV CLL will be cured by initial therapy with FCR [48, 49].

Patients who become refractory to chemoimmunotherapy and those with high-risk disease markers such as del(17p) or unmutated *IGHV* typically have less durable responses to chemoimmunotherapy. Several approaches have been used to manage these high-risk patients. The monoclonal antibody alemtuzumab (Campath I-H, anti-CD52) was shown to have a high rate of response in patients with del(17p) CLL and was found to be particularly useful in patients with the bulk of disease in the blood and bone marrow and minimal lymphadenopathy [50]. High-dose methylprednisolone was shown to induce a high rate of remission in high-risk patients and was most useful in those with bulky lymphadenopathy [51, 52]. The UK group attempted to combine these two approaches in their CLL206 trial, which gave concurrent alemtuzumab and methylprednisolone to TP53-deleted CLL [53]. Although their response rates, PFS, and OS were substantial for this population, the toxicity of this highly immunosuppressive approach was also apparent, with grade 3–4 infections occurring in half of the patients.

Given the significant unmet medical need for patients with high-risk CLL, particularly those who became refractory to chemoimmunotherapy, there was an urgent need for the development of novel agents to treat this disease. Years of laboratory research had revealed that two of the “Achilles heels” of CLL pathophysiology were the B-cell receptor (BCR) pathway and the intrinsic mitochondrial pathway of apoptosis, with its key anti-apoptotic protein B-cell leukemia/lymphoma-2 (BCL-2). The first effort to target the BCR pathway in CLL examined the Syk inhibitor fostamatinib. CLL patients treated with fostamatinib had significant shrinkage of lymphadenopathy while at the same time showing a rising lymphocyte count, which was initially concerning for disease progression [54]. Fostamatinib also had several off-target effects which led to significant toxicities and the development of this drug in CLL did not move forward. Shortly after this, two drugs entered into clinical trials which would forever change the history of CLL treatment, ibrutinib, the oral inhibitor of Bruton’s Tyrosine Kinase (BTK) [55], and idelalisib [56], an oral inhibitor of the delta-isoform of phosphoinositide-3-kinase (PI3K), which is selectively expressed in lymphocytes. In early-phase clinical trials, both of these drugs demonstrated an initial lymphocytosis and as with fostamatinib, at the same time nodal disease and cytopenias were improving. This phenomenon was later named “lymphocyte redistribution,” as it was recognized that through disruption of integrin signaling in the stromal microenvironment, CLL cells were being released from the lymph nodes and marrow and into the blood of patients and then subsequently undergoing apoptosis. The recognition that this epiphenomenon was not disease progression but quite the opposite—a sign of disease response—led to a consensus statement that lymphocyte redistribution should not constitute progression and a new category of response known as partial response with lymphocytosis (PR-L) or nodal response should be used to classify these patients [57].

Both ibrutinib and idelalisib were found to be highly active in early-phase trials, and remarkably patients had equivalent response rates irrespective of traditional high-risk markers such as del(17p) or unmutated IGHV [58, 59]. In phase III randomized trials, ibrutinib was found to have an overall survival benefit compared to the anti-CD20 antibody ofatumumab, leading to its FDA approval in relapsed/refractory

CLL [60]. Idelalisib plus rituximab was found to have an OS benefit compared to rituximab alone, leading to its approval in relapsed/refractory CLL [61]. Ibrutinib went on to receive frontline approval for CLL based on an overall survival benefit demonstrated in a phase III study comparing it to chlorambucil for initial therapy in older, frail patients [62]. Idelalisib was also being studied in several frontline trials in CLL and other indications, but due to an increased risk of mortality due to infectious complications as well as significant autoimmune toxicities, its development for frontline CLL therapy came to a halt in 2016 [63].

The other key pathway for CLL cell survival is the intrinsic mitochondrial pathway of apoptosis. CLL is well known to express high levels of the anti-apoptotic protein BCL-2, on which the malignant cells depend for their survival. An early attempt to target this pathway was the oral BCL-2/BCL-XL/BCL-w inhibitor navitoclax [64]. Although substantial efficacy was seen with navitoclax in CLL, there was also significant thrombocytopenia observed, which was thought to be on target toxicity due to inhibition of BCL-XL, which is important for platelet survival [65, 66]. A second-generation BCL-2 inhibitor was developed known as venetoclax, which is highly selective for BCL-2 with minimal activity against BCL-XL [67]. In the phase I first in human study, venetoclax was found to be so active against CLL cells that there were several cases of tumor lysis syndrome including one mortality due to this [68]. After lowering the initial starting dose and doing a gradual intrapatient dose ramp-up, the rates of tumor lysis syndrome dropped substantially and there were no further clinical sequelae. As with the BCR inhibitors, venetoclax induced a high rate of response even in patients refractory to fludarabine or harboring ominous prognostic markers such as del(17p). Unlike the BCR inhibitors, the responses induced by venetoclax included a significant number of patients achieving complete response with some patients achieving minimal residual disease negativity. A subsequent pivotal phase II study of venetoclax in patients with del(17p) confirmed these initial results [69] and led to FDA approval of venetoclax for relapsed/refractory CLL with del(17p) in 2016.

Although an exciting development, the novel therapies recently developed for CLL are unlikely to be curative on their own and therefore must be continued for ongoing therapy. Current clinical trials are looking at combination strategies to facilitate time-limited therapy, in some cases with curative intent. These promising trials involve novel agents plus chemotherapy, novel agents plus a monoclonal antibody, and novel agent-only combination approaches, all of which appear to be promising.

To date, the only consistently curative strategy for CLL has been allogeneic bone marrow transplantation. Many CLL patients are older and frail with significant comorbidities and do not tolerate myeloablative conditioning regimens well. Non-myeloablative conditioning regimens (also known as reduced-intensity conditioning) for transplantation have substantial efficacy with less toxicity, and appear to be equally effective for patients with high-risk disease [70]. Rates of success for allotransplant appear to be highest in patients who are maximally cytoreduced prior to transplant. Overall there is about a 40% rate of long-term progression-free survival in CLL patients treated with reduced-intensity allogeneic transplantation, although rates of graft vs. host disease and treatment-related mortality remain substantial. To build on this immunologic approach to treating CLL with less toxicity than allotransplant, several groups have recently presented data on a novel approach known as chimeric antigen receptor (CAR) T cell therapy. This approach utilizes autologous T cells from the patient and ex vivo introduces a CD19 antigen receptor. The T cells are then reinfused back into the patient where they can proceed to eradicate residual CLL. Several dramatic responses have been observed in refractory CLL patients treated with CAR-T cells, and some of these responses have had substantial longevity [71]. Toxicities include a cytokine release syndrome and neurotoxicity. Ongoing studies of this promising new approach in CLL will help define the optimal use of this technology in CLL.

Although there have been many exciting developments in CLL research over the last several years, there are several areas of unmet medical needs. These include patients with high-risk disease such as del(17p) or complex cytogenetics, whose responses to novel agents are less durable, patients who develop Richter's syndrome for which therapy remains inadequate, and patients who desire time-limited therapy with curative potential. Many clinical trials have launched recently to answer these questions, and these promising new approaches suggest that the future outcomes for patients with CLL will only continue to improve in time.

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Epidemiology and Etiology of Chronic Myeloid Leukemia

2

Maren Rohrbacher and Joerg Hasford

Introduction

Epidemiology can be defined as the study of the frequency, distribution, and causes of diseases in populations, mainly with observational designs. In recent years the traditional spectrum of epidemiology was extended to a variety of so-called hyphenated epidemiological subdisciplines like genetic, environmental, pharmaco, and healthcare epidemiology. Epidemiology has become an important multidisciplinary science and is essential for the identification of risk factors, assessment of the burden of disease, development of preventive actions, and promotion of public health. Chronic myeloid disease has been identified as a disease entity for the first time in 1845 independently by Virchow and Bennett [1, 2]. In 1973, Rowley published that the so-called Philadelphia (Ph) chromosome, which is pathognomonic for Ph-positive (Ph+) chronic myeloid leukemia (CML), is the result of a translocation between the chromosomes 9 and 22 [3]. From a commonly fatal disease CML has become, with the introduction of tyrosine kinase inhibitors (TKIs) since 2001, for most patients a kind of chronic disease. More recent studies report that the life expectancy of patients with CML approximates the one of the normal population [4, 5].

As CML is a very rare disease and the diagnosis requires elaborate diagnostic tools like cytogentic tests, FISH, or PCR techniques, it remained outside of the focus of most cancer registries and epidemiologists. Thus, reliable epidemiological information on Ph/BCR-ABL-positive CML is still limited, but much better compared to the situation 15 years ago. The aim of this chapter is to review the current knowledge regarding etiology, incidence, prevalence and survival rates, secondary malignancies, health care, and issues of the daily CML practice.

Etiology

CML is caused by a chromosome abnormality, the BCR-ABL fusion oncogene [Philadelphia chromosome (Ph)] [6, 7]. This oncogene is imperative for a Ph/BCR-ABL-positive CML. Specific genetic or environmental factors can result in the fusion of breakpoints of chromosome 9 in the ABL gene with certain breakpoints on chromosome 22 in the BCR gene [7].

No evidence for genetic predisposition in individual persons has been provided, and case reports of familial CML are rare [7–12]. Lifestyle factors such as smoking and a high body mass index have been identified as possible risk factors for CML [13–16].

An association between chemical exposure to benzene, organic solvents, alkylating agents, topoisomerase II inhibitors or other chemotherapeutic agents, and de novo CML has been shown repeatedly, but the evidence was not entirely consistent [7, 17–21]. An increased CML incidence has been seen among workers exposed to benzene or benzene-containing solvents [17, 20]. Benzene itself is not considered genotoxic, but its major hepatic metabolites, phenol, hydroxyquinone, and 1,2,4-benzenetriol and their metabolic products (e.g., 1,4-benzoquinone and semiquinone) are suspected to induce DNA damages in bone marrow cells as well as alkylating agents and topoisomerase inhibitors [22–25]. The benzene metabolite, trans-muconaldehyde, although genotoxic, seemed to get inactivated in the liver by glutathione. Thus, the active form could not reach sufficient levels in the marrow to harm hematopoietic cell chromosomes [7]. In cell line cultures, benzene-related metabolites, alkylating agents, or topoisomerase II inhibitors caused abnormalities of chromosomes 5 (monosomy or del(5q31)), 7 (monosomy), and 8 (trisomy), often causally associated with secondary acute

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myelogenous leukemia after cytotoxic therapy [7, 17, 22–24, 26–28]. Neither relevant breaks in chromosomes 9 or 22 nor the formation of the BCR-ABL fusion gene were observed in in vitro tests [7, 22, 24, 27–29]. Studies of late effects of chemotherapy for a variety of malignancies did not show an increased risk for secondary CML [7].

In comparison to chemical exposures, leukemogenic effects of acute, high-dose ionizing radiation exposure have been identified by the Atomic Bomb Casualty Commission in Japan after World War II in extensive epidemiological studies [20, 30–32]. The latest published data indicated that 20% of all leukemias were classified as CML based on an analysis between 1950 and end of 2001 [33, 34].

Also the incidence of CML among the Chernobyl cleanup workers increased in the last 20 years after the Chernobyl Nuclear Power Plant accident [35]. In the United States, CML represents about 17% of the potentially radiation-induced new cases of leukemia per year [7]. Biological plausibility further strengthened the compelling epidemiological evidence for radiation-induced CML by hematopoietic cell line cultures: The BCR-ABL oncogene could result from high-dose X-ray or gamma irradiation and the subsequent transcription of BCR-ABL message in such cells [36, 37]. High-dose radiation exposure can directly generate leukemia-specific fusion genes [37].

Incidence

Knowledge on clinical and molecular features of Ph/BCR-ABL-positive CML is extensive, but the epidemiology has still not been studied in detail [34, 38]. Sources of these data are mortality statistics [39], cancer population-based registries such as the Swedish Cancer Registry [40, 41], the Saarland Registry [42, 43] in Germany, the database of the Surveillance, Epidemiology and End Results (SEERs) Program of the United States National Cancer Institute [44, 45], or surveys such as the European Treatment and Outcome Study CML-Registry (EUTOS) [46].

Worldwide CML incidence rates vary from 0.6 to 2.8 cases per 100,000 inhabitants with an obvious increase in age [38, 40–52] (Table 2.1). CML occurs with greater frequency in men than in women as shown in male-to-female ratios ranging between 1.2 and 1.8 [34, 38, 40–47, 49, 53, 54].

The most recently published results from the EUTOS population-based CML-registry which collected incidence data from 20 European countries reported a raw incidence of 0.39/100,000/year in people 20–29 years old, and of 1.52 in those >70 years old, showing a maximum of 1.39 in Italy and a minimum of 0.69 in Poland [46]. Here, the median age at the time of diagnosis was about 56 (range 50–64) years. With a male-to-female ratio of 1.16 men were more often affected. Interestingly, a majority (53.5%) of the patients presented no palpable spleen at diagnosis, and 88.2% were classified as EUTOS score low-risk patients [46]. Some geographic and/or ethnic variations and different diagnostic accurateness might contribute to the variability of the reported incidences.

Table 2.1 Crude and standardized CML incidences of population-based registries and surveys

	Time of observation	Number of patients	Incidence crude	Incidence (WSP)[a]
SEER [44, 45]	1998–2000	–	–	1.8[b]
	2003–2007	4653	–	1.7[b]
	2008–2012	5955	–	1.8[b]
EUTOS registry[c] [46]	2008–2012	2904	1.0	0.9
France [51]	1985–2006	906	–	0.8
France [52]	1998–2009	781	–	0.8
Swedish Cancer Registry [40, 41]	1998–2000	260	1.0	0.7
	2001–2008	704	1.0	0.7
	2009–2014	633	1.1	0.7
Scotland Leukemia Registry[c] [48]	1999–2000	64	0.6	–
Thames Registry [47]	1999–2000	180	–	0.8
Leukemia Research Fund [49]	1984–1993	1115	–	0.6
Cancer Registry of Saarland [42, 43]	1998–2000	65	2.0	1.0
	2001–2007	142	1.9	0.9
	2008–2012	140	2.8	1.2
Southwest Germany[c] [54]	1998–2000	172	0.6	–
Southeast Germany [50]	2004	201	1.9	1.3

Modified with permission from Rohrbacher and Hasford [38]
[a]World Standard Population
[b]United States Standard Population
[c]CML cases with known Ph/BCR-ABL-positive status

A crude incidence of Ph/BCR-ABL-positive CML of 0.6 per 100,000 is available from the Scotland Leukaemia Registry [48] and from a survey in the Southwest of Germany [54] (Table 2.1). Both studies covered a population size of about 9 million inhabitants. In the German study the incidence of all reported 218 CML cases including negative and unknown Ph/BCR-ABL status was 0.8, and of CML and CMML (0.2) combined 1.0 [54]. As the Ph/BCR-ABL status was only available for 87.2% of the German CML patients and not for any of the 61 patients with a diagnosis of CMML, incidence estimates provided there probably represent the lower margin of the true CML incidence. The variations of incidences seen might indicate geographic and/or ethnic variability beyond technical artifacts [34, 38, 55]. Some registries try to increase data comparability by standardization according to the age structure of the world standard population (WSP). WSP weighs age-specific incidences in

populations with higher proportions of younger people than in the European standard population [56].

All publications considered in Table 2.1 are from northern Europe [40–43, 46–49, 54] or the United States [44, 45]. There seems to be variability of incidences of geographic areas even in the same country as exemplified by the Swedish National Cancer Registry [49] and the Goteborg Central Disease Registry [57] which reported incidences for polycythemia vera and primary myelofibrosis differing by a factor of up to more than two [38]. This is of interest, as differences in the CML risk group composition between southern (Italy) and northern European countries (Poland, the United Kingdom, Austria, Germany) were reported [46, 58, 59]. However, the recently observed data of the EUTOS registry could not identify a particular regional clustering of high and low incidences among Europe [46].

Between 1993 and 2004 the second Edition of the International Classification of Diseases for Oncology (ICD-O) (WHO Geneva 1990) for coding CML cases was commonly used, which did not differentiate true CML, Ph- and BCR-ABL-negative CML, chronic myelomonocytic leukemia (CMML), or subacute myeloid leukemia [38]. Since 2005, the discriminate coding of the molecular BCR-ABL-negative and -positive status has been possible on the basis of the new third Edition of ICD-O (WHO Geneva 2000) [38]. Basic data, stratified for BCR-ABL status, have not been shown in the latest cancer reports (Table 2.1). Consequently, the published incidences for CML may be higher than the true ones as BCR-ABL-negative cases are included. Up to 5% of patients with chronic myelogenous leukemia do not have the Ph-translocation t(9;22)(q34;q11) or a BCR-ABL molecular rearrangement [60].

Prevalence and Survival Rates

Prevalence of chronic, life-threatening, and costly disease has a major impact on the healthcare systems [61–63]. By the targeted treatment with TKIs a long-term survival is observed [62, 63]. An 8-year overall survival (OS) of 89% was recently published which approximates a normal life expectation [64]. Thus, this improved life expectation under the treatment of TKIs directly increases prevalence rates.

CML prevalence data itself remain scarce as population-based registries mostly provide “CML” data in a general category of “Leukemia” [41, 43, 45]. Nevertheless, some estimations are available from France, Sweden, the United States, and Germany: Within a French survey 906 newly diagnosed CML cases were identified in a population of about four million inhabitants for the period of 1985 to 2006 [51]. The calculated prevalence rate had increased from 5.8/100,000 during 1998 to 6.8 (2002) to 7.3 (2003) and to 10.4 in 2007. In another French survey based on 781 CML patients from population-based registries, the following data were presented [52]: The 5-year relative survival (RS) rates among patients with (Ph+) CML were 44% when diagnosed in 1980–1986, 64% in 1987–1999, and 89% in 2000–2009. The 8-year RS rate of patients with Ph+ CML diagnosed was 83% in 2000–2009.

For the Swedish population, prevalence data from 1985 to 2012 showed an increase from 3.9 to 11.9 per 100,000 inhabitants, and as an assumption 22.0 per 100,000 inhabitants by 2060 [5, 62]. The 5-year OS increased from 0.18 to 0.82, and the 5-year RS between 2006 and 2012 was close to a normal 40-year-old, but lower for 80-year-old CML patients (0.95 and 0.63).

For the US population the following data were available [63, 65, 66]: Estimated prevalence was about 70,000 CML patients in 2010, 112,000 in 2020, 144,000 in 2030, 167,000 in 2040, and 181,000 in 2050 against the backdrop of an annual incidence of 4800 CML patients [63]. A mortality ratio of CML patients (in their first 10 years since the diagnosis of CML) vs. the general population with the same age distribution was calculated to be 1.53 [63]. Using the SEERs database, the 5-year OS was improved for all patients between 2000 and 2005 [65]. Compared with patients who were diagnosed in 2000, the 5-year survival improved among patients aged 15–44 years (hazard ratio (HR) for mortality 0.43), aged 45–64 years (HR 0.716), and aged 65–74 years (HR 0.692), and patients aged 75–84 years had an increased 5-year OS rate from 19% in 2000 to 36% in 2005 (HR 0.568). These data were retrieved from 5138 patients [65]. SEERs data were also used to compare ethnic survival rates among Caucasian, African-Americans, and other races and within each race to see survival differences from the pre-imatinib (1973–2000) to post-imatinib eras (2002–2008) [66]. Here, the RS was significantly improved in the imatinib era, but these improvements were modest in the population-based data compared to those reported from clinical trials [66]. Interestingly, young (<50-year-old) female African-American CML patients have a lower RS in the imatinib era compared to young female Caucasian CML patients.

Considering the situation in Germany, the authors based their study on population-based data of about 10.5 million people in the statutory health insurance system in Bavaria for the years 2008–2013 [67]. Survival rates were adapted from the literature. The mean estimated age-standardized (Old European Standard Population) incidence rates per 100,000 inhabitants were 1.300 and 1.768 for women and men. Based on the population data, a total number of about 9000 CML patients was estimated in Germany for 2012, and it was expected that the number of CML patients would increase further until at least 2040 to 2050 with a maximum of more than 20,000 CML patients as the most probable scenario [67].

As a consequence of this considerable increase of the CML prevalence, the burden for the healthcare systems will rise with respect to costs and clinical services used.

Secondary Malignancy

The observed rise of the prevalence rates, associated with a long-term survival for the patients with TKI treatment, is still under investigation especially focusing on the risk of secondary malignancies [68, 69]. So far, a significant higher occurrence of secondary solid tumors such as gastrointestinal, nose, and throat cancer was observed in the imatinib era than in the pre-imatinib era [69].

Population-based data from 868 CML patients diagnosed between 2002 and 2011 in the Swedish Cancer registry and from 5511 CML patients in the SEERs database indicate that CML patients treated in the TKI era had an increased risk of developing a second (non-hematological) malignancy compared to the general population [68, 69]. In detail, the risk of second malignancies was higher in the CML cohort ($N = 868$) compared to the general population, with a standardized incidence ratio (SIR) of 1.52 (95% CI 1.13–1.99). Here, the SIR before and after the second year following diagnosis of CML was 1.58 and 1.47 [68]. Among the 8511 adult CML patients, 446 patients developed 473 secondary malignancies. The SIR for secondary malignancies in CML patients was significantly higher with the observed/expected ratio 1.27 ($P < 0.05$) and absolute excess risk of 32.09 per 10,000 person years compared to the general population [69]. The rate of secondary malignancies of all sites in the post-imatinib era was significantly higher compared to the pre-imatinib era with the observed/expected ratio of 1.48 versus 1.06 ($P = 0.03$) [69]. The authors assumed that the estimated elevated risk is more likely linked to the underlying CML disease than to the TKI therapy [68].

The occurrence of these secondary cancer entities in TKI-treated CML patients in comparison to the general population is the focus of ongoing research.

Health Care and Daily Practice

Patient's survival depends on the available national healthcare system [70–77]. There, aspects of medicines' costs play an important role in treatment decisions, and may also be the reason behind different treatment recommendations in different countries [77]. The growing life expectancy is in addition an influencing factor for the daily clinical practice. Especially, an age disparity for elderly patients in the administration of TKI therapy has been observed as an issue.

For the approved TKIs such as imatinib, dasatinib, and nilotinib, there are high healthcare costs noticed [76, 77]. In the United States the price for imatinib has been increased threefold from initially $30,000 per year in 2001 to $92,000 per year in 2012 [76, 77]. At the same time in 2012, three new drugs were approved by the US Food and Drug Administration (FDA) with pricing levels at $28,000 (omacetaxine), $118,000 (bosutinib), and $138,000 (ponatinib) per year.

The reason for the high TKI therapy prices can be the expensive developmental costs for new targeted, effective, safe, and tolerable medicines, but the negative clinical aspects of the pricing have also to be discussed. A forum of CML experts emphasized that this economic model of high therapy costs can cause a comprised access of needy patients to the targeted drugs with the clinical consequence of a less effective therapy and a poorer survival outcome [77]. Additionally, this pricing model can potentially harm the sustainability of healthcare systems [77].

Examples of the pharmacoeconomic/epidemiologic associations between national healthcare systems, patient's individual access to targeted CML treatment, and patients' survival rates are shortly described in the following: US patients have to pay an average of 20% of the drug prices out of their own pocket (about $20,000–$30,000 per year) which may induce a personal bankruptcy depending on the private household budget. These circumstances can be reasons for poor compliance, drug discontinuation, and worse outcome: It was observed that 10% of the US patients failed to take prescribed drugs mostly due to costs. By evaluating the survival rates in the United States, the estimated 5-year survival rate is currently still 60% although there was an improvement since the imatinib release in 2001 [77]. This suggests a lower TKI treatment penetration rates in the United States compared to higher TKI treatment penetration and compliance rates in Europe, e.g., in Sweden with an estimated 10-year survival rates of 80% where patients have an access to lifesaving CML drugs via the national healthcare policy [77].

Consequently, there are further discussions needed whether lowering the TKI prices would improve treatment penetration rates, increase compliance, and expand CML patient population living longer and continuing on TKI therapy. This concept would paradoxically increase revenues to pharmaceutical companies from sales of TKIs [77].

Focusing on the clinical practice, CML experts from, e.g., the European LeukemiaNet (ELN) (http://www.leukemia-net.org) have developed recommendations for the medical management of patients of all ages with CML including the definition of CML phases, the appropriate use of TKIs, the evaluation of cytogenetic and molecular responses, and optimized treatment strategies [78–82]. The patient's age at the time of diagnosis and the individual concomitant comorbidities are factors influencing treatment decisions. It was observed that especially elderly patients received either a reduced dose of imatinib in case of concomitant comorbidities or another therapy but not the targeted TKI treatment with the benefit of a better

survival [80–84]. This was confirmed by the validated EUTOS score demonstrating that under TKI treatment increasing age did not play a significant negative role for the probability to achieve a complete cytogenetic remission and a long progression-free survival [85, 86].

However, based on published data, there seems to be an age disparity in imatinib-containing treatments being probably associated with a worse survival for especially elderly CML patients: The use of imatinib was inversely associated with patient's age: 90, 75, and 46% for patients aged 20–59, 60–79, and ≥80 in a study based on SEERs and population-based data with 423 CML patients diagnosed in 2003 [84]. Elderly patients who received imatinib survived significantly longer than those who did not. After adjusting for the patient's age, the imatinib use did not vary significantly by race/ethnicity, socioeconomic status, urban/rural residence, presence of comorbid conditions, or insurance status [84].

Further examples for age disparity in CML treatment: In Lithuania, survival data and the TKI treatment penetrance from 601 CML patients were analyzed [87]. The patients' data were retrieved from the national haematological disease-monitoring system being diagnosed between 2000 and 2013 [87]. The reported median age at diagnosis was 62 years. A 5-year RS rate increased from 0.33 (95% CI, 0.27–0.40, in 2000–2004) to 0.55 (95% CI, 0.47–0.63, in 2005–2009), but the 5-year RS survival rates for patients aged 65–74 years and ≥75 years were only 0.33 (95% CI, 0.24–0.42) and 0.18 (95% CI 0.07–0.23). The TKI penetrance rate for the patients grew from 1.5% (in 2000–2004) to 30.6% (in 2005–2009) and 69.1% (in 2010–2013); however, the TKI penetrance rate was lower in the older age groups (60% for the 65–74, and 19% for the ≥75 years patient group, in 2010–2013). Hence, the RS for elderly patients remained poorer as this patient population rarely received TKIs for their CML treatment [87].

In Sweden, the cancer registry provided data from 779 CML patients for an observed period between 2002 and 2010 [5]. The population's median age here was 60 years. Regarding the TKI penetration rate, nearly 50% of the patients received a TKI, a proportion that increased to 94% for younger (<70 years) and 79% for older (>80 years) patients during 2007–2009. The estimated 5-year RS was close to 1.0 for patients younger than 60 years, 0.9 for those aged 60–80 years, and only 0.6 for elderly patients (>80 years) [5]. Despite respective treatment recommendations, the majority of elderly CML patients received non-imatinib-containing regimens as first-line treatment during 2002–2004. From 2006 a change in imatinib therapy for elderly patients was observed, but nevertheless, this population was less likely to be treated with TKIs than younger patients.

Besides the described TKI treatment age disparity in daily practice, it is known that elderly patients are underrepresented in most investigational clinical trials. This was shown in a comparison between multicenter trials and population-based registries during the last decades [38]: The median age in multicenter trials [58, 88–109] (Table 2.2b) is 49 years, even in trials without age limitation as an inclusion criterion [89, 95, 104]. In contrast, the median age is up to 67 years in population-based registries [44–46, 110–112] (Table 2.2a), concluding that data of clinical trials underestimate the true age of the CML population. Within a German survey it was determinated that the chance for a CML patient <65 years to be enrolled in a clinical study was 3.8 times higher than for a CML patient ≥65 years [54].

Table 2.2 Age at diagnosis of CML patients in population-based registries and clinical trials

	Number of cases	Age (years), mean ± SD/ median (range)
(A) Registries [Ref. No.], data period		
EUTOS registry, 2002–2006 [46]	2904	56 (18–99) tbc
Czech Republic/Slovakia CAMELIA registry, 2000–2008 [110]	661	51 (15–83)
Thames Cancer Registry, UK, 1999–2000 [47]	180	65 (20–98)
Austrian CML registry, <2000 to 2009 [111]	179	53 (17–88)
SEER Cancer Statistics Review, 2003–2007 for whites [44]	4653	Median age 67 (n.a.)[a]
SEER Cancer Statistics Review, 2008–2012 for whites [44]	5955	Median age 66 (n.a.)
SEER Cancer Statistics Review, 1973–1998 for all races [112]	8229	Median age 64 (n.a.)
SEER Cancer Statistics Review, 2008–2012 for all races [45]	7441	Median age 64 (n.a.)
(B) Clinical Trials [Ref. No.]		
The Italian Cooperative Study Group on Chronic Myeloid Leukemia, N Engl J Med 1994 [90]	322	48 ± 14
Hehlmann et al., Blood 1994 [83]	513	48 (17–85)
Allan et al., Lancet 1995 [59]	587	47 (15–84)
Guilhot et al., N Engl J Med 1997 [108]	754	50 (7–70)
Hasford et al., JNCI 1998 [109]	1303	49 (10–85)
The Benelux CML Study Group, Blood 1998 [91]	195	56 (20–83)
Bonifazi et al., Blood 2001 [106]	317	49 (9–73)
Baccarani et al., Blood 2002 [105]	538	45 ± 13
Hehlmann et al., Leukemia 2003 [88]	534	48 (10–83)
Kluin-Nelemans et al., Blood 2004 [92][b]	407	60 (20–81)

(continued)

Table 2.2 (continued)

	Number of cases	Age (years), mean ± SD/ median (range)
Druker et al., N Engl J Med 2006 [107]	1106	50 (18–70)
Kantarjian et al., Blood 2006 [93]	929	48/43 (15–84)
Hehlmann et al., Blood 2007 [89]	621	49 (11–90)
Jabbour et al., Blood 2009 [94]	169	50 (17–94)
Saussele et al., Blood 2010 [95][c]	84	37 (16–62)
Palandri et al., Haematologica 2010 [96]	495	49 (18–80)
Efficace et al., Blood 2011 [97]	448	57 (19–87)
Hanfstein et al., Leukemia 2012 [98][c]	1223	52 (16–85)
Marin et al., J Clin Oncol 2012 [99]	282	46 (13–86)
Cortes et al., J Clin Oncol 2012 [100]	502	48 (18-91)
Jabbour et al., Leukemia 2013 [101]	315	58 (21–85)
Castagnetti et al., Annals of oncology 2015 [102]	2784	50 (18–87)
Saussele et al., Blood 2015 [103][c]	1519	63 (16–88)

Modified with permission from Rohrbacher and Hasford [38]
[a]n.a. = range not available
[b]This study comprises mostly elderly patients since younger patients were recruited for transplantation studies
[c]The referenced publications [95, 98, 103] retrieved their data from the CML IV Study

Another practical aspect in daily CML management is the association between the frequency of molecular monitoring and the risk of progression and progression-free survival (PFS). This was evaluated in a US study with 402 CML patients being on first-line imatinib therapy [113]. There, it has been shown that patients who had on average three to four qPCR tests per year had a lower risk of progression and longer PFS compared to patients who had no qPCR monitoring. These results were also observed in in those patients being monitored with one to two qPCR tests per year compared to those without any molecular monitoring [113]. Thus, patients can benefit from regularly scheduled qPCR testings which is in accordance to current CML recommendations.

Furthermore, it seems that patients treated in teaching hospitals achieve better outcomes: Based on 1491 patients of the German CML Study IV, the authors compared the outcomes of patients from teaching hospitals with those from municipal hospitals and office-based physicians [114]. Adjusting for age, EUTOS score, Karnofsky performance status, year of diagnosis, and experience with CML, a significant survival advantage for teaching hospital patients (HR 0.63–0.61) was found. In particular, when treated in teaching hospitals, patients with blast crisis showed a superior outcome (2-year survival rate: 47.7% vs. 22.3% vs. 25.0%) [114].

Some of the current issues in CML health care and daily clinical practices have been highlighted. More efforts are necessary that all CML patients get access to the targeted TKI treatment and benefit from the reported better survival associated with regular molecular monitoring. Especially, for the population of elderly patients the age disparity in TKI-containing treatment and the exclusion in investigational trials should be reduced by continuous education and implementation of the proposed recommendations.

Summary

Epidemiological information on Ph/BCR-ABL-positive CML is still limited. Available CML incidence rates vary from 0.6 to 2.8 cases per 100,000 inhabitants with an obvious increase in age. Some geographic and/or ethnic variations might contribute to this variability, but there are also differences in diagnostic accurateness. The observed prevalence rates increase with the widespread use of TKIs improving patients' survival and quality of life. Consequencently, an estimated 8-year OS of 89% has recently been determinated indicating an almost normal life expectation for the CML patients.

This outstanding therapeutic achievement asks for further research on the occurrence of secondary malignancies, other therapy-related risks, the treatment of elderly patients, the economic impact on healthcare systems by the expensive long-term treatment, and the chances to stop treatment with TKIs in patients with complete remission without risking relapse of CML.

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3 Pathology of the Chronic Myeloid Leukemias

Barbara J. Bain

Introduction

The chronic myeloid leukemias are a group of hematological neoplasms resulting from mutation in either a pluripotent (lymphoid-myeloid) stem cell or in a multipotent myeloid progenitor or stem cell. The former group of leukemias are actually, or potentially, of mixed phenotype while the latter have a purely myeloid phenotype.

Diagnosis of these conditions is based on clinical and hematological features with genetic analysis being crucial for the recognition of some categories. Genetic analysis has been incorporated, as far as current knowledge permits, into the classification of the chronic myeloid leukemias, in the 2016 update of the 2008 World Health Organization (WHO) *Classification of Tumours of Haematopoietic and Lymphoid Tissues* [1]. Depending on their typical hematological features and any relevant genetic abnormality, they are assigned to the categories: (a) myeloproliferative neoplasm; (b) myelodysplastic/myeloproliferative neoplasm; and (c) myeloid or lymphoid neoplasm with rearrangement of *PDGFRA*, *PDGFRB*, or *FGFR1*or with *PCM1-JAK2*.

In making a morphological diagnosis, the peripheral blood features are often of critical importance. The bone marrow aspirate and trephine biopsy sections give useful supplementary information. Flow cytometric immunophenotyping is only of diagnostic importance when there is presentation with acute-phase disease or when acute transformation subsequently occurs. Immunohistochemistry on trephine biopsy sections can be useful, particularly in the recognition of dysplastic megakaryocytes and for confirmation of the presence of dysplastic mast cells. Cytochemistry is now of little importance in diagnosis; the neutrophil alkaline phosphatase score is redundant when genetic analysis is available and immunophenotyping is superior to cytochemistry for the recognition of the lineage involved in acute transformation.

Chronic Myelogenous Leukemia

Chronic myelogenous leukemia (CML) results from a mutation in a pluripotent hematopoietic stem cell that leads to formation of a *BCR-ABL1* fusion gene [2]. The associated acquired cytogenetic abnormality is usually t(9;22)(q34.1;q11.2), but there are other mechanisms, including variant and complex translocations (see Chap. 6). The derivative chromosome 22 is known as the Philadelphia (Ph) chromosome. Alternative designations of this condition are chronic granulocytic leukemia and chronic myeloid leukemia, but it should be noted that the latter term, although very widely used and now favored by the WHO, is open to misinterpretation since it is also used as a generic term for all the chronic myeloid leukemias.

This disease occurs at all ages, but incidence increases steadily with age and is somewhat higher in men than in women. Common clinical features are weight loss, low-grade fever, sweating, splenomegaly, and, when disease is advanced, hepatomegaly [3]. Lymphadenopathy is only a feature when acute transformation occurs. Patients with a very high white cell count can have features of leukostasis, such as blurred vision and priapism. Many patients are now diagnosed as a result of an incidental blood count when they are asymptomatic.

The natural history of CML is that the chronic phase of the disease is followed by acute transformation (myeloid, lymphoid, or mixed), which may be preceded by an accelerated phase. The abrupt onset of acute transformation without a preceding accelerated phase is more common in lymphoid transformation.

Peripheral Blood Count and Cytology

The peripheral blood count in *chronic-phase CML* typically shows leukocytosis and anemia. Usually there is also

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thrombocytosis although the platelet count can be normal or reduced. Some patients have thrombocytosis without leukocytosis. Such *BCR-ABL1*-positive cases should be recognized as a variant of CML [2]. The blood film characteristically shows a particular increase in myelocytes and neutrophils (Fig. 3.1). Basophils are almost invariably increased and eosinophils usually so [4]. Eosinophil and basophil myelocytes may also be present. The absolute monocyte count is increased but not in proportion to the increase in granulocytes. The number of blast cells and promyelocytes is proportionate to the number of myelocytes. Cells of the granulocyte lineages do not show dysplastic features. Platelets show anisocytosis with some giant forms. There may be occasional circulating megakaryocyte nuclei with scanty cytoplasm ("bare" megakaryocyte nuclei). Red cells may show nonspecific abnormalities, such as anisocytosis and mild poikilocytosis. There may be circulating nucleated red blood cells. Neutrophil alkaline phosphatase is reduced in the great majority of patients, but this test is redundant when cytogenetic and molecular analyses are available.

Peripheral blood features of the *accelerated phase* of CML may include leukocytosis or thrombocytosis that is refractory to treatment; an increasing basophil count; a disproportionate increase in blast cells; the appearance of dysplastic features such as hypolobulated neutrophils and circulating micromegakaryocytes; thrombocytopenia; and increasing anemia. A disproportionate increase in eosinophils can occur but is much less common than marked basophilia. Poikilocytosis may become more marked and there may be teardrop poikilocytes.

In *blast transformation* there is usually an increase in blast cells in the peripheral blood (Fig. 3.2). Usually these are myeloblasts, megakaryoblasts, or lymphoblasts. When transformation is megakaryoblastic, there may also be circulating micromegakaryocytes. Less common forms of transformation (determined by the underlying further mutations that have occurred) include mixed phenotype, monoblastic, eosinophilic, erythroblastic, and hypergranular promyelocytic.

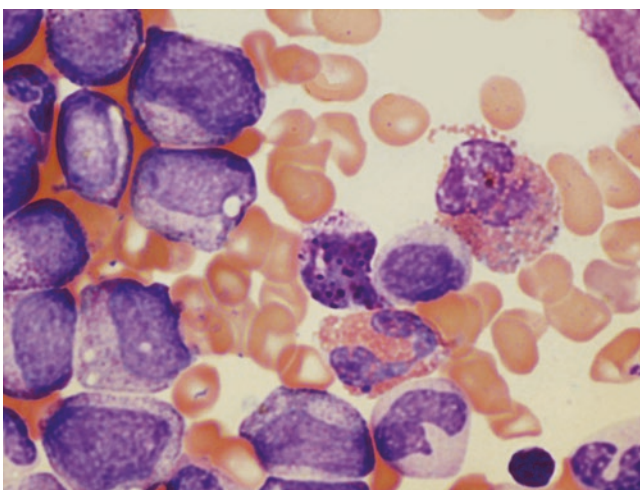

Fig. 3.1 Peripheral blood (PB) film from a patient with chronic myelogenous leukemia with an unusually high white cell count showing an increase of neutrophils, eosinophils, basophils, and granulocyte precursors. May-Grünwald-Giemsa stain (MGG), high power

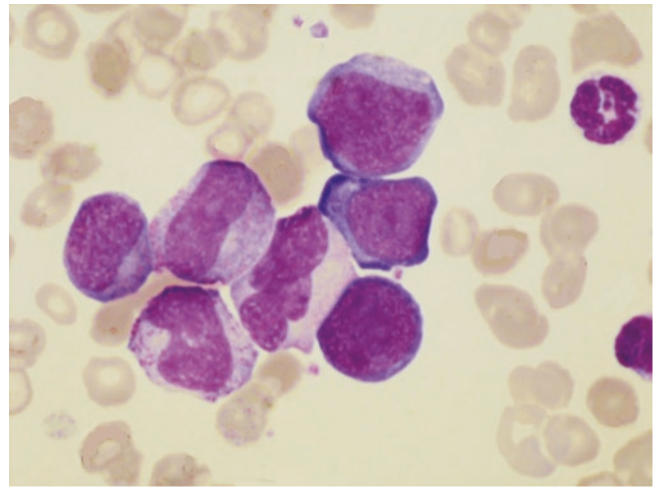

Fig. 3.2 PB film from a patient with transformation of chronic myelogenous leukemia showing three blast cells, granulocyte precursors, and several dysplastic cells of neutrophil lineage. MGG, high power

Bone Marrow Cytology

The bone marrow aspirate in *chronic-phase CML* shows marked hypercellularity due to an increase in granulocytes and their precursors (Fig. 3.3). The myeloid:erythroid ratio is almost always greater than 10:1 and often of the order of 25:1. Megakaryocytes are usually increased with a tendency to be smaller than normal with reduced nuclear lobulation, reflecting a decrease in ploidy. However, micromegakaryocytes, such as those seen in the myelodysplastic syndromes, are not a feature of chronic-phase disease. Sometimes there is an increase in storage cells—pseudo-Gaucher cells and sea blue histiocytes.

In the *accelerated phase*, the aspirate may show increased blast cells, increased basophils, and dysplastic features in the cells of any lineage.

In *blast transformation*, the bone marrow shows increased blast cells, except in the minority of cases in which transformation is first detected at an extramedullary site. Myeloblasts are often agranular and Auer rods are usually absent. In the case of myeloid or mixed lineage transformation, there are usually dysplastic features, particularly in the megakaryocyte lineage (Fig. 3.4).

Flow Cytometric Immunophenotyping

Immunophenotyping is not diagnostically useful in chronic-phase disease.

Genetics

Cytogenetic and genetic features are discussed in Chap. 6.

Histology

In *chronic-phase CML*, trephine biopsy sections show an increase in cells of all granulocyte lineages but, apart from the loss of fat cells, with retention of normal bone marrow architecture (Fig. 3.5) [5–7]. There is an expansion of the band of myeloblasts and promyelocytes that is usually detected against the bony spicule and around arterioles. Eosinophils are readily detected, but basophils are not specifically identifiable on hematoxylin and eosin (H&E) stains as granules are dissolved during processing. Megakaryocytes are increased in number with a reduction in average size and nuclear lobulation. They are normally located and do not form large clusters. Erythropoiesis is decreased. Mast cells and plasma cells are often increased. Increased storage cells may be apparent (Fig. 3.6). Bone marrow vascularity is increased (neoangiogenesis). Reticulin is usually normal or only mildly increased.

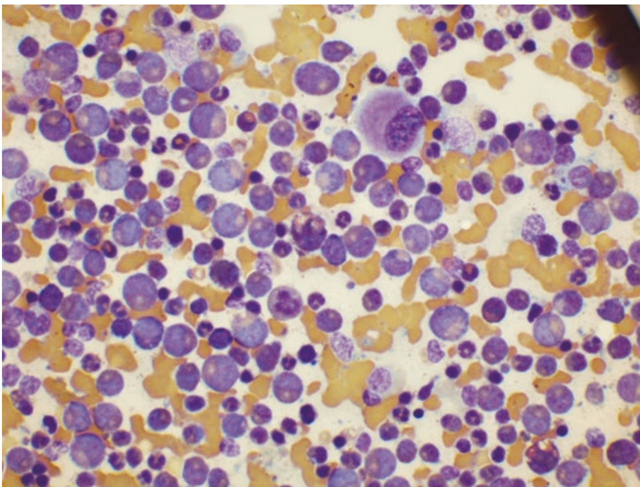

Fig. 3.3 Bone marrow film from a patient with chronic myelogenous leukemia showing increased granulopoiesis and a hypolobated megakaryocyte. MGG, low power

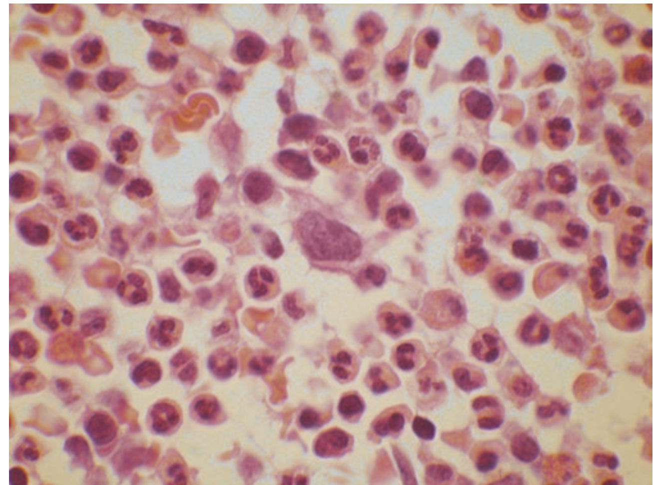

Fig. 3.5 Bone marrow trephine biopsy section from a patient with chronic myelogenous leukemia showing increased granulopoiesis and a relatively small, hypolobated megakaryocyte. Hematoxylin and eosin stain (H&E), low power

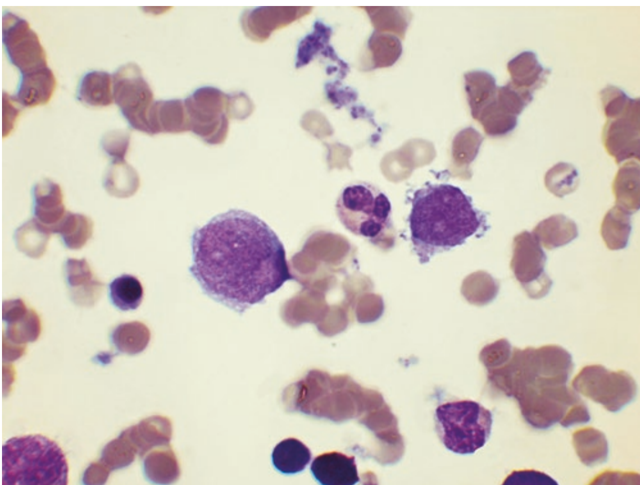

Fig. 3.4 Bone marrow film from a patient with megakaryoblastic transformation showing a neutrophil flanked by a blast cell (*left*) and a micromegakaryocyte (*right*). MGG, high power

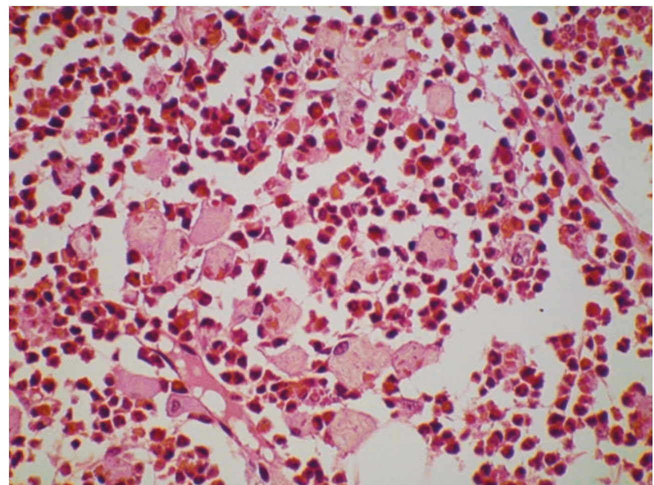

Fig. 3.6 Bone marrow trephine biopsy section from a patient with chronic-phase myelogenous leukemia showing numerous pseudo-Gaucher cells. H&E, low power

In *accelerated phase*, the changes that would be expected from the bone marrow aspirate are present. In addition, the bone marrow architecture may be abnormal, e.g., with megakaryocytes located adjacent to bony spicules or forming large clusters. Intravascular hemopoiesis and bone marrow necrosis can occur. Reticulin may be increased. Sometimes the increase in reticulin is marked and there is also collagen fibrosis and osteosclerosis (Fig. 3.7).

Immunohistochemistry with CD42b or CD61 monoclonal antibodies can be used to identify dysplastic megakaryocytes. CD34 antibodies can identify blast cells and endothelial cells of new vessels.

In *blast transformation*, there is an increase in the blast cells of one or more lineages. In myeloid transformation, there can also be a marked increase of dysplastic megakaryocytes, often in large clusters or sheets. The pattern of blast infiltration may initially be random focal, but subsequently blast cells obliterate maturing hematopoietic cells. Reticulin and collagen fibrosis are common, particularly when there is an increase in megakaryoblasts and dysplastic megakaryocytes.

Immunohistochemistry can be used to identify myeloblastic crisis (CD68, lysozyme), megakaryoblastic crisis (CD42b, CD61), erythroblastic crisis (antiglycophorin—CD235a or CD236R), and B-lymphoblastic crisis (CD79a is more generally positive than CD20). CD34 immunohistochemistry can help in the quantification of blast cells.

In extramedullary transformation, there is initially infiltration of another tissue or organ, e.g., a lymph node, with subsequent spread to the marrow. In extramedullary transformation, immunohistochemistry is useful for confirmation of the diagnosis.

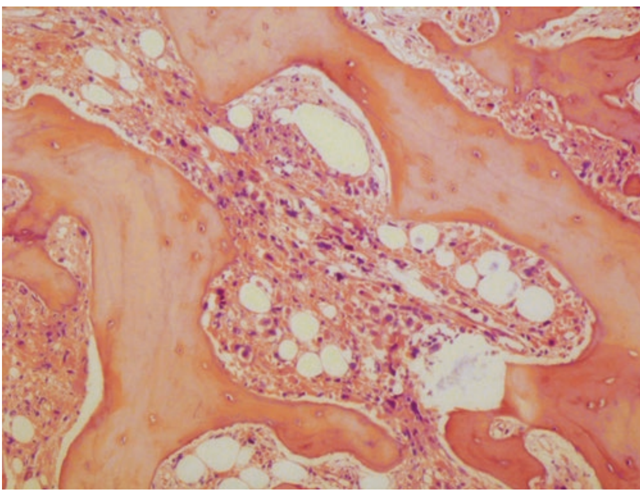

Fig. 3.7 Bone marrow trephine biopsy section from a patient with accelerated phase of chronic myelogenous leukemia showing myelofibrosis and osteosclerosis. There are numerous dysplastic megakaryocytes embedded in the loose fibrous tissue. H&E, low power

Atypical (Ph-Negative) Chronic Myeloid Leukemia

Atypical chronic myeloid leukemia (aCML) is an uncommon, Ph-negative, *BCR-ABL1*-negative chronic myeloid leukemia, which is categorized in the WHO classification as an MDS/MPN [8–10]. Clinical features are similar to those of CML, but the prognosis is worse. Death may result from bone marrow failure or evolution to acute myeloid leukemia (AML). Atypical CML is mainly a disease of adults, particularly elderly adults with a similar incidence in men and women.

Peripheral Blood Count and Cytology

The peripheral blood shows leukocytosis and anemia (Fig. 3.8). The WHO classification has a white cell count (WBC) of at least 13×10^9/L as a diagnostic criterion. There may be anisocytosis, poikilocytosis, macrocytosis, or dimorphism. The platelet count is often reduced but may be normal or increased. In comparison with CML, anemia tends to be more severe and thrombocytopenia more common. There is an increase in neutrophils and their precursors. Eosinophils and basophils are often increased but less consistently than in CML. Basophils are usually less than 2% of leucocytes. However, some patients have prominent eosinophilia. The monocyte count is relatively higher than in CML; it may be more than 1×10^9/L, but monocytes are less than 10% of leucocytes. Granulocyte precursors are also present. In comparison with chronic myelomonocytic leukemia, promyelocytes, myelocytes, and metamyelocytes are at least 10% of leukocytes and

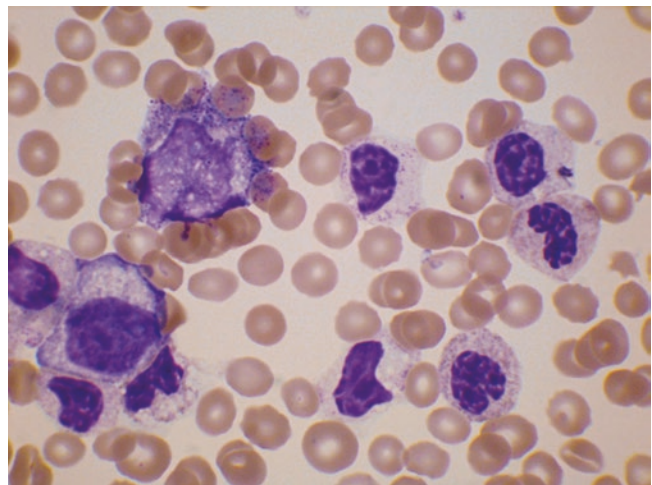

Fig. 3.8 PB film from a patient with atypical chronic myeloid leukemia showing neutrophil precursors and dysplastic neutrophils (hypolobated, nuclear clumping, and hypogranularity). MGG, high power

sometimes 15% or higher. These may include blast cells but, by definition, blast cells (plus promonocytes) are less than 20% in the blood (and the bone marrow). Dysplastic features are present in neutrophils; hypolobation, abnormal nuclear shapes, increased chromatin clumping, and reduced granularity may be seen. Monocytes may also be dysplastic, showing hyperlobation or hypolobation, increased cytoplasmic basophilia, and increased granularity. The neutrophil alkaline phosphatase score is variable and is not diagnostically useful.

Bone Marrow Cytology

The bone marrow aspirate shows increased cellularity with an increase mainly in neutrophils and their precursors. Monocytes and their precursors may be increased and a nonspecific esterase stain can help in their detection. Megakaryocytes may be present in normal numbers or may be decreased or increased. There is dysplasia, which is often of trilineage. Dysgranulopoiesis is usual, but there may also be ring sideroblasts and other features of dyserythropoiesis, hypolobated megakaryocytes, and micromegakaryocytes.

Flow Cytometric Immunophenotyping

Immunophenotyping is not known to be diagnostically useful.

Genetics

Karyotypic abnormalities are common and can include trisomy 8, 20q–, and i(17q) and abnormalities of chromosomes 12, 13, 14, 17, and 19. *SETBP1*, *ETNK1*, *NRAS*, *KRAS*, *CBL*, and *TET2* may be mutated. *CSF3R* mutation has been reported but its presence favors a diagnosis of chronic neutrophilic leukemia. Patients with *BCR-ABL1,* rearrangement of *PDGFRA*, *PDGFRB*, or *FGFR1*, or *PCM1-JAK2* are specifically excluded from this diagnostic category.

Histology

Cellularity is increased as a result of an increase in neutrophils and precursors and a variable increase in monocyte precursors. There is dysplasia and the architecture is disorganized. Reticulin may be increased and collagen fibrosis and osteosclerosis occasionally occur. Immunohistochemistry is useful to highlight dysplastic megakaryocytes (CD42b and CD61) and increased monocytes (CD14 and CD68R).

Chronic Myelomonocytic Leukemia

This is Ph-negative chronic myeloid leukemia that is categorized in the WHO classification as an MDS/MPN [11, 12]. The most prominent clinical features are anemia and splenomegaly, but skin and lymph node infiltration and pleural, peritoneal, and pericardial effusions can also be seen. It is mainly a disease of the middle aged and elderly and shows a male predominance. Transformation to AML occurs in up to a quarter of the patients.

Peripheral Blood Count and Cytology

The peripheral blood shows a normocytic or macrocytic anemia. Red cells are sometimes dimorphic. Leukocytosis is usual but not invariable. There is monocytosis with, by definition, a monocyte count of more than 1×10^9/L (Fig. 3.9). Neutrophils may be increased, normal, or decreased. Neutrophil precursors may be present but, in contrast to aCML, they are less than 10% of the cells and usually less than 5%. There may be small numbers of blast cells and promonocytes; by definition, they total less than 20% of leukocytes, but they are usually much less. The number of blast cells (plus promonocytes) in the blood is of prognostic significance and the presence of 5% or more leads to a classification as CMML-2. A minority of patients have prominent eosinophilia. The platelet count is often reduced but can be normal or high.

Dysplastic features may be present, but are usually less prominent than in aCML. Monocytes may be immature (cytoplasmic basophilia, reduced chromatin condensation, or reduced nuclear lobulation).

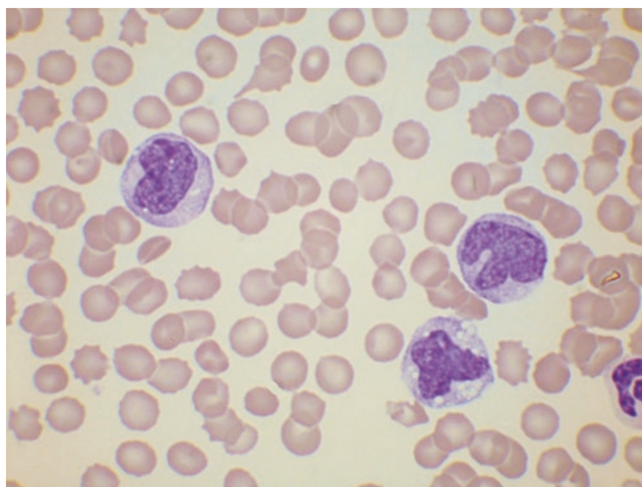

Fig. 3.9 PB film from a patient with chronic myelomonocytic leukemia showing thrombocytopenia and three immature, abnormal monocytes. MGG, high power

Bone Marrow Cytology

The bone marrow shows increased cellularity due to an increase of neutrophils and monocytes and their precursors but a nonspecific esterase stain may be necessary to demonstrate the increase in cells of monocyte lineage. Blasts plus promonocytes are less than 20%. The number of blast cells (plus promonocytes) in the marrow is of prognostic significance and the presence of 10% or more leads to the classification as CMML-2. Auer rods are rarely present but, when present, also lead to classification as CMML-2; a myeloperoxidase or Sudan black B stain is useful for their detection. Some patients have an increase of eosinophils and precursors. There is variable dysplasia, which may include ring sideroblasts.

Flow Cytometric Immunophenotyping

Immunophenotyping may show monocytes to be phenotypically abnormal with reduced, increased, or aberrant expression of various antigens. An abnormal phenotype may be the result of immaturity of monocytes (e.g., reduced CD14 expression) or of aberrant antigen expression (e.g., expression of CD2).

Genetics

Karyotypic abnormalities are detected in a quarter to a half of patients. They include trisomy 8, monosomy 7 and 7q–, and rearrangements with a 12p breakpoint. Common molecular changes include mutations of *RAS* group genes, *RUNX1*, *TET2*, *SRSF2*, *ASXL1*, *SETBP1*, and *CBL*. By definition, *BCR-ABL1*, rearrangement of *PDGFRA*, *PDGFRB* and *FGFR1*, and *PCM1-JAK2* are absent.

Histology

Trephine biopsy sections show the changes that would be expected from the aspirate. Hypercellularity is usual and results from an increase in the cells of both neutrophil and monocyte lineages. Immunohistochemistry (CD14, CD68R, or CD163) may be necessary to demonstrate the increase in the cells of monocyte lineage. Erythropoiesis may be quantitatively normal or increased. There may be nodules of plasmacytoid dendritic cells, confirmed by immunohistochemistry for CD4, CD14, CD68R, and CD123 [13, 14]. Reticulin deposition is often increased.

Chronic Eosinophilic Leukemia

Chronic eosinophilic leukemias, in the WHO classification, are categorized either as chronic eosinophilic leukemia (CEL), not otherwise specified [15], or as chronic myeloid or lymphoid neoplasm associated with rearrangement of *PDGFRA*, *PDGFRB*, or *FGFR1*, or with *PCM1-JAK2* [16–18]. Diagnosis and categorization require both cytogenetic and molecular analyses, the latter to detect the most frequent rearrangement of *PDGFRA*, a *FIP1L1-PDGFRA* fusion gene resulting from a cryptic deletion at 4q12. Diagnosis of CEL requires an eosinophil count of at least 1.5×10^9/L and some evidence that the process is leukemic in nature, such as a clonal cytogenetic or molecular abnormality or an increase in blast cells.

Leukemias with rearrangement of *PDGFRA* can present as CEL, AML with eosinophilia, or T-lineage acute lymphoblastic leukemia (ALL) with eosinophilia [17, 19]. Leukemias with rearrangement of *PDGFRB* can present as CEL or as either CMML or aCML with eosinophilia. Leukemias with rearrangement of *FGFR1* can present as CEL, AML with eosinophilia, or acute lymphoblastic leukemia/lymphoma (ALL) with eosinophilia; ALL is most often of T lineage but can be of B lineage [17]. Leukemias with *PCM1-JAK2* can be CEL or aCML but some patients have had the features of primary myelofibrosis [18]. In all these disorders, patients who present with chronic phase disease can subsequently suffer acute transformation.

Clinical presentation can be with features suggestive of leukemia (such as anemia, splenomegaly, and sometimes lymphadenopathy) or features reflecting tissue damage by eosinophils (such as cardiac, respiratory, and neurological symptoms). CEL associated with *FIP1L1-PDGFRA* shows a remarkable male predominance. Cases with *PCM1-JAK2* also show a marked male predominance [18]. CEL associated with *PDGFRB* or *FGFR1* rearrangement is also more common in males. CEL associated with rearrangement of *PDGFRA* or *PDGFRB* is sensitive to imatinib and making these specific diagnoses is therefore important. Cases with *PCM1-JAK2* show some responsiveness to ruxolitinib.

Elevation of serum vitamin B_{12} and serum tryptase is usual in patients with CEL associated with *FIP1L1-PDGFRA*.

Peripheral Blood Count and Cytology

The peripheral blood shows an increase in eosinophils, which may be cytologically fairly normal or may show reduced or increased nuclear lobulation, loss of granules, darkly staining (purple) granules, or cytoplasmic vacuolation (Fig. 3.10). There may be eosinophil precursors and blast cells. By definition, blast cells are less than 20%. Some patients also have an increase in neutrophils or monocytes, anemia, or thrombocytopenia.

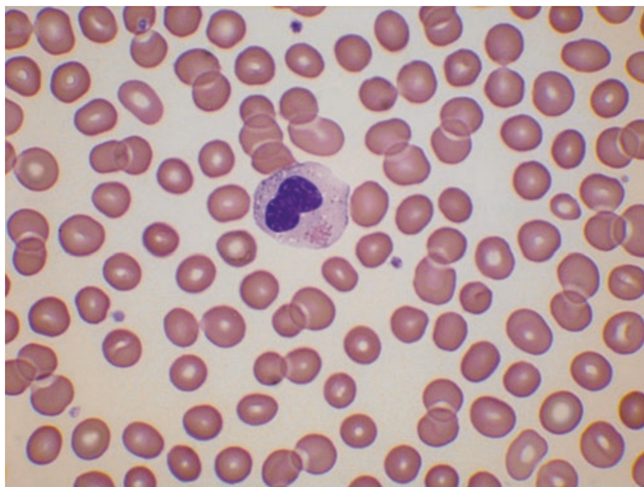

Fig. 3.10 PB film from a patient with chronic eosinophilic leukemia associated with *FIP1L1-PDGFRA* fusion showing an eosinophil with a poorly lobulated nucleus, a few vacuoles, and only a small cluster of granules. MGG, high power

Bone Marrow Cytology

The bone marrow shows an increase in eosinophils and their precursors plus a variable increase in precursors of monocytes and neutrophils. Blast cells may be increased but are less than 20%.

Flow Cytometric Immunophenotyping

Immunophenotyping is occasionally needed to show the lineage of blast cells and thus distinguish CEL and related conditions from ALL with reactive eosinophilia. It must be noted that in the case of CEL associated with rearrangement of *PDGFRA* or *FGFR1*, there may be either presentation as ALL or a lymphoblastic transformation; usually but not invariably the lymphoid component is of T lineage.

Genetics

Karyotypic analysis may show t(5;12)(q31~q33;p12) or another translocation with a 5q31-32 breakpoint involving *PDGFRB*. Rarely there is a translocation with a 4q12 breakpoint involving *PDGFRA*, but usually rearrangement of this gene is cryptic, resulting from an interstitial deletion. *FGFR1* rearrangement most often results from t(8;13)(p11;q12), leading to a *ZMYM2-FGFR1* fusion gene. *PCM1-JAK2* fusion results from the t(8;9)(p22;p24.1) translocation. Other nonspecific chromosomal abnormalities may be found including trisomy 8, 20q–, monosomy 7, and i(17q). The demonstration of t(8;21)(q22;q22.1), inv(16)(p13.1q22), or t(16;16)(p13.1;q22) excludes a diagnosis of CEL and leads to a diagnosis of AML regardless of the blast percentage.

The most important molecular abnormality that must be sought in suspected CEL is the *FIP1L1-PDGFRA* fusion gene, which can be demonstrated either by fluorescence in situ hybridization (FISH) analysis or by PCR (nested PCR often being needed) [16]. Occasionally patients with *FIP1L1-PDGFRA*-associated CEL develop a further mutation in the fusion gene, sometimes associated with imatinib resistance or transformation to AML [19].

Histology

Trephine biopsy sections show the expected increase in eosinophils and precursors and a variable increase in the cells of neutrophil or monocyte lineages. Charcot–Leyden crystals are also sometimes seen [20]. Cases associated with rearrangement of *PDGFRA* or *PDGFRB* may show an increase of mast cells, which are sometimes spindle shaped and clustered. Their presence can be highlighted by immunohistochemistry for mast cell tryptase. The mast cells may show aberrant expression of CD25 and sometimes of CD2, whereas the neoplastic cells of systemic mastocytosis usually show aberrant expression of both CD2 and CD25.

Chronic Neutrophilic Leukemia

Chronic neutrophilic leukemia is a rare myeloproliferative neoplasm that occurs mainly in adults [21]. It is characterized by increased neutrophil production, splenomegaly, and sometimes hepatomegaly. Transformation to AML can occur and at this stage the neutrophil count may fall [22]. Older adults are mainly affected with no gender difference.

Peripheral Blood Count and Cytology

There is leukocytosis and neutrophilia (Fig. 3.11). The WHO classification requires a white cell count of at least 25×10^9/L for this diagnosis. Neutrophils may be heavily granulated and Döhle bodies are sometimes present, but there are no dysplastic features and there are only small numbers of neutrophil precursors [23]. With disease progression there may be anemia and thrombocytopenia.

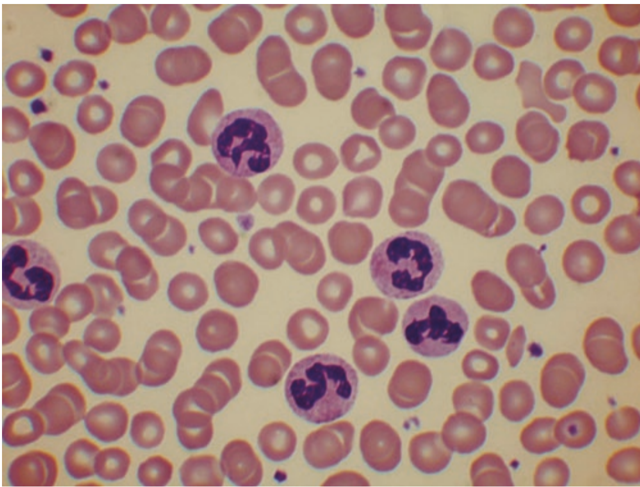

Fig. 3.11 PB film from a patient with chronic neutrophilic leukemia showing cytologically normal neutrophils. MGG, high power

Bone Marrow Cytology

The bone marrow aspirate shows an increase of neutrophils and their precursors without dysplastic features or any increase in the blast cells. The bone marrow must be carefully examined to exclude a plasma cell neoplasm since reactive neutrophilia due to multiple myeloma or monoclonal gammopathy of undetermined significance is an important differential diagnosis [23].

Flow Cytometric Immunophenotyping

Immunophenotyping has no role in diagnosis.

Genetics

Cytogenetic analysis is usually normal. A minority of patients have karyotypic abnormalities typical of myeloid neoplasms such as trisomy 8, trisomy 9, trisomy 21, 11q–, 12p–, or 20q–. Nullisomy 17, a complex karyotype and several nonrecurrent translocations have also been reported. Sometimes an initially normal karyotype becomes abnormal, with acute transformation [22]. There is a strong association with an activating mutation in the proximal membrane domain of *CSF3R* [24]. There is also often an associated mutation of *SETBP1* or *ASXL1*. Occasional patients have a *JAK2* V617F mutation, which may be homozygous [23]. By definition, there is no *BCR-ABL1* fusion gene.

Histology

Trephine biopsy sections show increased granulopoiesis.

Juvenile Myelomonocytic Leukemia

This is a rare myelodysplastic/myeloproliferative neoplasm of children [25–29]. Peak incidence is under the age of 3 years and the condition is twice as common in boys. Predisposing conditions include neurofibromatosis type 1 (*NF1* mutated), Noonan syndrome (*PTPN11* mutated), and *CBL*-mutation associated syndrome. Clinical features can include fever, cough, splenomegaly, hepatomegaly, often lymphadenopathy, skin lesions (an eczematous or maculopapular rash or xanthomas), and a bleeding tendency. Respiratory tract infections are common. In patients with underlying neurofibromatosis there may be café-au-lait spots whereas patients with Noonan syndrome have facial dysmorphism and congenital cardiac anomalies. A hallmark of the disease is increased sensitivity in vitro to granulocyte-macrophage colony-stimulating factor as a result of increased signaling through the RAS-MAPK pathway. The rate of disease progression is quite variable, but prognosis is generally poor unless hematopoietic stem cell transplantation is carried out. Transformation to AML occurs in about 15% of the patients [28]. Occasional patients have had transformation to B-cell precursor ALL and the same acquired genetic lesion has sometimes been found in T-lymphoid and myeloid cells, suggesting that the leukemic clone may be derived from a pluripotent lymphoid-myeloid stem cell [28].

Peripheral Blood Count and Cytology

The peripheral blood shows leukocytosis, monocytosis, and neutrophilia with a lesser increase in granulocyte precursors (Fig. 3.12). Eosinophilia and basophilia are less common than neutrophilia. WHO criteria include a monocyte count of at least 1×10^9/L. There is a variable degree of dysplasia. Blast cells (plus promonocytes) are usually

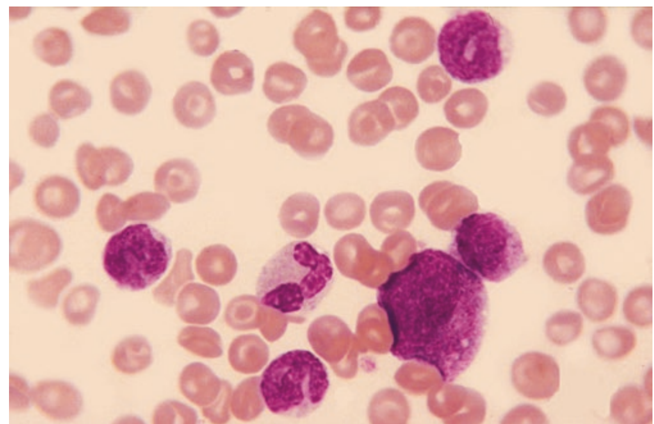

Fig. 3.12 PB film from a patient with juvenile myelomonocytic leukemia showing a neutrophil, a promyelocyte, and dysplastic cells, mainly of monocyte lineage. MGG, high power

low and, by definition, never more than 20% in the blood or bone marrow. Anemia and thrombocytopenia are usual. There may be circulating nucleated red blood cells or neutrophil precursors and some patients have macrocytosis. There is often increased rouleaux formation.

Blood tests show other abnormalities. Polyclonal hypergammaglobulinemia is common and the erythrocyte sedimentation rate is increased. There may be autoantibodies including anti-erythrocyte antibodies. The hemoglobin F percentage is usually increased in comparison with age-matched healthy children and there may be other features suggesting reversion to fetal-type erythropoiesis, such as increased expression of the i antigen and decreased expression of the I antigen, carbonic anhydrase, and hemoglobin A_2.

Bone Marrow Cytology

The bone marrow is hypercellular as a result of increased granulopoiesis. Blast cells (plus promonocytes) are less than 20%. Megakaryocytes are often reduced.

Flow Cytometric Immunophenotyping

Immunophenotyping is not diagnostically useful.

Genetics

There is no specific chromosomal abnormality and cytogenetic analysis is often normal. Some patients have monosomy 7, trisomy 8, or a complex karyotypic abnormality. Chromosomal analysis may be initially normal but become abnormal during the course of the illness. Genetic analysis shows four nonoverlapping groups of patients with loss-of-function mutation in *NF1* (inherited or acquired) or mutation in *PTPN11* (inherited or acquired) [30], *CBL* [31], or a *RAS* group gene (*NRAS* or *KRAS*) [32]; a mutation in one of these genes is found in 85% of cases. In children with neurofibromatosis there may be homozygosity for the mutant gene as a result of acquired uniparental disomy or there may be somatic mutation in the initially normal allele [33, 34]. Similarly, patients with an inherited *CBL* mutation usually show loss of heterozygosity.

Histology

The bone marrow is hypercellular as a result of increased granulopoiesis and a variable increase in monocytopoiesis and erythropoiesis. Reticulin may be increased.

Conclusions

The chronic myeloid leukemias are a heterogeneous group of hematopoietic neoplasms, some with mainly proliferative features and others with myelodysplastic/myeloproliferative characteristics. Origin may be in a pluripotent lymphoid-myeloid stem cell or in a committed myeloid cell. Since leukemias with a *BCR-ABL1* fusion gene or rearrangement of *PDGFRA* or *PDGFRB* are sensitive to tyrosine kinase inhibitors, precise diagnosis is of considerable importance.

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Molecular Biology and Cytogenetics of Chronic Myeloid Leukemia

4

Marina Konopleva, Alfonso Quintás Cardama, Hagop Kantarjian, and Jorge Cortes

Introduction

Chronic myeloid leukemia (CML) is a clonal myeloproliferative neoplasia characterized by the t(9;22)(q34;q11) balanced reciprocal translocation that causes the fusion of a portion of chromosome 9 to chromosome 22 (der22), thereby replacing a fragment of chromosome 22 which fuses to chromosome 9 (der9). The resultant minute chromosome der22, designated as the Philadelphia chromosome (Ph), is the hallmark of CML [1]. The molecular event resulting from this translocation is the hybrid *BCR-ABL1* oncogene, which encodes the constitutively active BCR-ABL1 protein kinase [1]. The BCR-ABL1 oncoprotein can transform cells through phosphorylation of tyrosine residues on a variety of intermediary proteins that transmit signals from the cytoplasm to the nucleus. The ultimate proof that BCR-ABL1 kinase expression can induce CML was provided by experiments in which murine bone marrow was transfected with a retrovirus encoding *BCR-ABL1* and transplanted into irradiated syngeneic recipients. Transplanted recipients developed several hematologic malignancies, most frequently a myeloproliferative syndrome that resembles very closely chronic-phase CML [2]. The demonstration that BCR-ABL1 kinase activity played a critical role in cellular transformation provided the rationale for developing molecules aimed at targeting such activity. Kinase-based assays demonstrated that imatinib, the first tyrosine kinase inhibitor (TKI) developed for the treatment of CML, potently inhibited ABL1 kinase [3, 4]. This inhibitory activity translated into impressive clinical activity [5]. The remarkable clinical success of imatinib propelled the rational design and development of other TKIs (e.g., nilotinib, dasatinib, bosutinib, ponatinib) aided by structural biology and high-throughput medicinal chemistry methods. Despite these agents' clinical activity, many patients with CML receiving TKI therapy frequently manifest measurable amounts of residual disease, and in some the TKI therapy eventually fails. Among patients with accelerated-phase (AP) or blastic phase (BP) CML, responses are less frequent and often short-lived [6]. These shortcomings of TKI therapy have resulted in research efforts aimed at understanding the behavior of CML stem cells, the molecular basis of transformation to AP and BP, and the mechanisms of resistance to TKIs.

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The *BCR-ABL1* Oncogene

The breakpoints within *ABL1* map either upstream of exon Ib, downstream of exon Ia, or, more frequently, between exons Ib and Ia [7]. In most patients with CML and in one-third of adults with Ph-positive B-cell acute lymphoblastic leukemia (Ph + B-ALL), the breakpoints within *BCR* map to a 5.8-kb area spanning exons e12–e16 (formerly b1–b5), referred to as the *major breakpoint cluster region* (M-*bcr*). Alternative splicing produces fusion transcripts with either e13a2 or e14a2 junctions that give rise to a 210 kDa protein ($p210^{BCR\text{-}ABL1}$) [8]. In two-thirds of adults with Ph + B-ALL and in occasional cases of CML, the *BCR* breakpoint localizes to a 54.4-kb area between exons e2′ and e2 (*minor breakpoint cluster region* or m-*bcr*), which produces an e1a2 transcript that translates into $p190^{BCR\text{-}ABL1}$ and is associated with a more aggressive CML course. A third breakpoint cluster region (μ-*bcr*) gives rise to a 230 kDa fusion protein ($p230^{BCR\text{-}ABL1}$) that is associated with a very indolent course of CML [9].

Several experimental models, such as *BCR-ABL1*-expressing CD34+ cells in culture [10, 11] or retrovirally transduced *BCR-ABL1*-positive mouse cells [2, 12], have helped establish a direct causality between BCR-ABL1 and CML. A lysine-to-arginine substitution at residue 1176 (K1176R) in the ATP-binding pocket of ABL1 inactivates

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the kinase activity of BCR-ABL1 and prevents the development of leukemia in mice, even when *BCR-ABL1*^{K1176R} is expressed in hematopoietic stem cells (HSCs) [13]. Further proof of the central role of *BCR-ABL1* as the pathogenetic driver in CML was provided by developing transgenic mice in which the tetracycline-responsive element (tet-O) inducibly drives *BCR-ABL1* expression specifically in HSCs. SCL-tTA/BCR-ABL-tetO mice, resulting from crossing *BCR-ABL1* tet-O mice with mice expressing the tetracycline transactivator (tTA) under the control of the murine stem cell leukemia (SCL) gene 3′ enhancer, developed a myeloproliferative disease that recapitulated multiple features of human CML upon tetracycline withdrawal [14]. The clinical success of imatinib, a small molecule that inhibits the kinase activity of BCR-ABL1, has further confirmed the oncogenic role of this protein kinase.

Autoregulation of the *BCR-ABL1* Protein Kinase

BCR-ABL1 kinase is a multidomain protein (Fig. 4.1). The N-terminus of BCR-ABL1 includes the “Cap” region, present in two different isoforms, 1a and 1b, as a consequence of alternative splicing of the first exon. The ABL1b isoform contains a C_{14} myristoyl moiety covalently linked to the N-terminus and is expressed at higher levels than type 1a, which is not myristoylated. ABL1 also contains highly conserved Src-homology-2 (SH2) and SH3 domains and a tyrosine kinase domain [15]. The SH2 domain interacts with and phosphorylates signaling proteins, including p62dok, c-Cbl, Rin-1, Tub, and mDab1. Mutations within the SH2 domain have been found to delay the onset of, but fail to prevent, BCR-ABL1-induced myeloproliferation [16]. The last ABL1 exon region contains several distinct domains, including four proline-rich SH3 motifs (which act as docking sites for SH3 domains of adaptor proteins such as Crk, GRB2 [growth-factor-receptor-bound 2], and Nck) [17, 18], a DNA-binding domain, an actin-binding domain, three nuclear localization signals, and one nuclear export signal, which determines the subcellular localization of ABL1 (Fig. 4.1). BCR also exhibits a complex spatial modularity that includes a coiled-coil oligomerization domain, a serine/threonine kinase domain, a Dbl/CDC24 guanine–nucleotide exchange factor homology domain, a pleckstrin homology domain, a calcium-dependent lipid-binding site, and a RAC guanosine triphosphatase-activating protein domain. Tyr177 at BCR serves as docking site for GRB2, GRB10, 14-3-3, and ABL1 proteins through its SH2 domain [19]. The myristoyl modification at the end of the N-terminal segment of ABL1b engages the C-terminal lobe of the ABL1 catalytic domain and facilitates the docking of the SH2 and SH3 domains onto the kinase domain [20, 21]. The absence of the myristoyl group results in constitutive tyrosine kinase activity [15]. In the inactive conformation of the kinase domain, the rotation of the helix αC displaces critical catalytic residues out of the active site, thus hampering ATP access to the active site [15]. The X-ray crystal structure of the oligomerization domain of BCR-ABL1 (residues 1–72 or BCR_{1-72}) showed that two monomers associate in an antiparallel

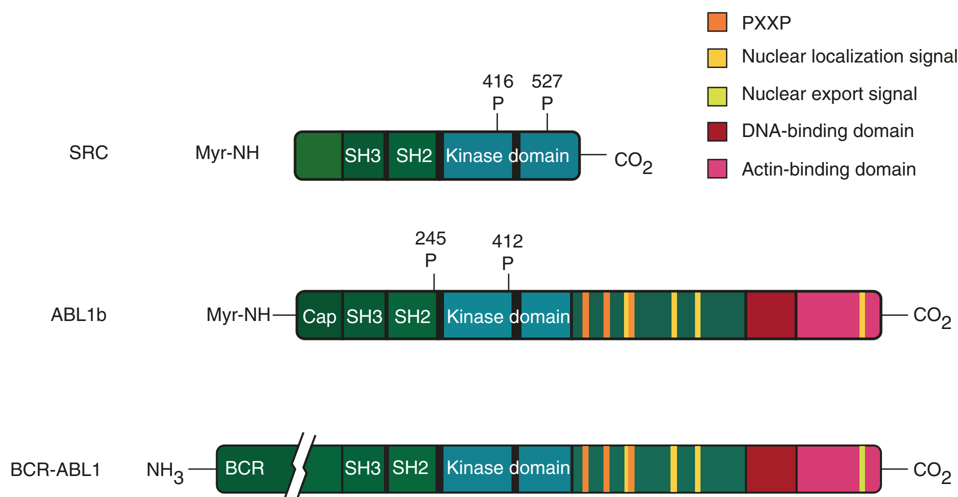

Fig. 4.1 Modular structure of the BCR-ABL1 protein. The modular structure of BCR-ABL1 is lined up against those of SRC and ABL1b kinases, which share a common central core that includes a tyrosine kinase domain, a SRC-homology-2 (SH2) domain, and an SH3 domain. The NH2 terminus in ABL1 and BCR-ABL1 kinases is the “Cap” region. Alternative splicing of the first ABL1 exon yields two ABL1 isoforms (a and b). ABL1b contains a myristate site (Myr-NH) at the extreme end of the amino-terminal segment, which binds to the kinase domain and keeps the SH2–SH3 autoinhibitory structure in place (i.e., in the “off state”)

dimer, which in turn stacks to form a tetramer [22]. Mutations at the coiled-coil domain impairing BCR-ABL1 oligomerization drastically compromise the kinase activity and the leukemogenic potential of BCR-ABL1 [23].

Signaling Pathways Stemming from BCR-ABL1 Kinase

BCR-ABL1 activates numerous downstream signaling pathways (Fig. 4.2). Such activation stems from a multiprotein complex formed by BCR-ABL1 kinase and an array of substrates and adaptor proteins, which include GRB2, CrkL, c-CBL, and p62(DOK). Signaling emanating from this complex leads to cellular transformation. The phosphorylation of the tyrosine residue 177 (Tyr177) of BCR is essential for BCR-ABL1-mediated leukemogenesis [24]. A tyrosine-to-phenylalanine substitution at this residue (Tyr177Phe) impairs GRB2 binding and markedly diminishes BCR-ABL1-induced RAS activation [25], which impedes the transformation of primary bone marrow cultured in the presence of ABL1 kinase activity [25]. Transfection of a mutant *BCR-ABL1* isoform carrying the Tyr177Phe mutation prevents the induction of a myeloproliferative disorder in a murine stem cell transplantation model of CML [25]. Tyr177 functions as a high-affinity docking site for the SH2 domain of GRB2, which in turn recruits SOS (a guanine–nucleotide exchanger of *RAS*), resulting in activation of RAS [19] and the scaffold adapter GRB2-associated binding protein 2 (GAB2) [19]. The importance of GAB2 is illustrated by the fact that BCR-ABL1 fails to transform primary myeloid cells from *GAB2*$^{-/-}$ mice [26]. The GRB2/GAB2 complex activates phosphatidylinositol 3-kinase (PI3K)/AKT [27] and ERK in primary CML cells. Interestingly, MEK-ERK activation is cytokine dependent in chronic phase (CP) but becomes constitutively activated in CML BP and is readily detectable in CD34+ progenitors [28]. Mutation of BCR-ABL1 protein at any of three of the direct binding sites for GRB2, namely CBL, p62(DOK), and CRKL, resulted in defective transformation of primary hematopoietic cells in a mouse model of CML because of decreased activation of the MAP kinase and PI3K pathways but not of the transcription factor signal transducer and activation of transcription 5 (STAT5) [29]. Overall, these data indicate that disruption of the interaction between BCR-ABL1 and BCR-ABL1 substrates may result in abrogation of leukemogenesis.

Other important proteins phosphorylated by BCR-ABL1 kinase are the SRC family kinases (SFKs) HCK, LYN, and FGR. LYN kinase is activated indirectly upon activation of JAK2 kinase by BCR-ABL1. Inhibition of JAK2 reduces the level of the SET protein and increases serine/threonine phosphatase 2A (PP2A) and tyrosine phosphatase 1 (SHP1) activities, which decrease the levels of activated LYN [30]. In turn, phosphorylated HCK recruits STAT5 [19, 31], which upregulates cyclin D1. This in turn induces cell cycle progression from G_1 to S

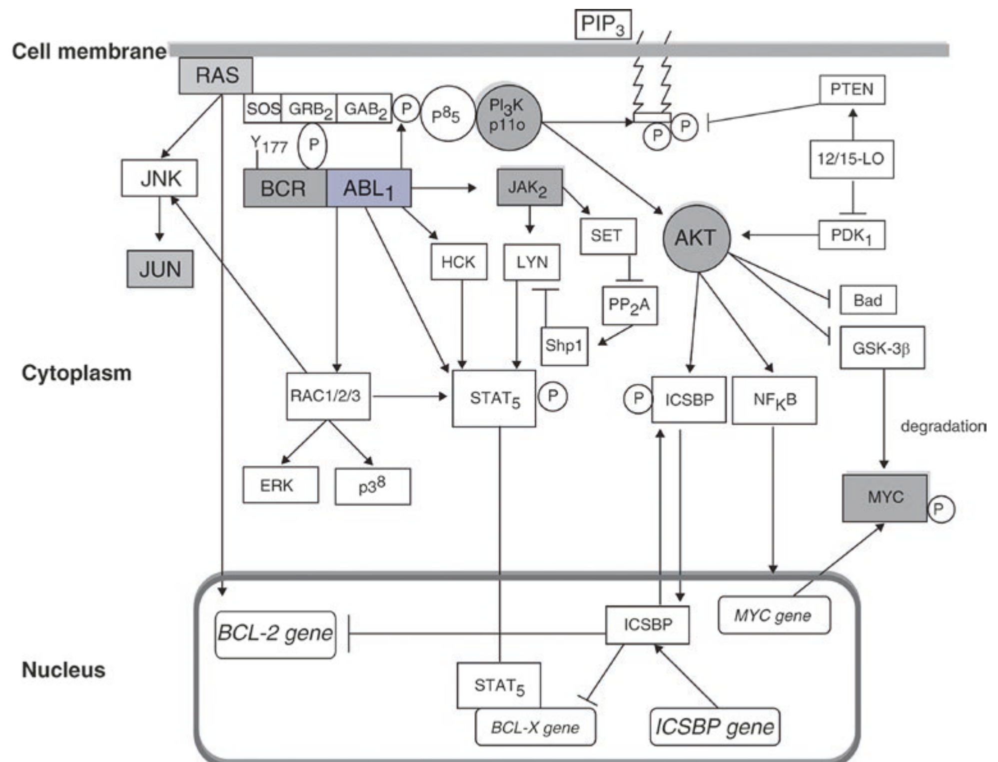

Fig. 4.2 Signaling pathways stemming from BCR-ABL1 kinase. BCR-ABL1 kinase phosphorylates a series of intracellular substrates, thus activating several signaling pathways that promote cell proliferation and inhibition of apoptosis

phase [32]. However, whether Src kinases are indispensable for BCR-ABL1-mediated leukemogenesis remains controversial, since *BCR-ABL1* can cause a CML-like picture in bone marrow of mice lacking *Lyn, Hck, and Fgr* [33]. In turn, the generation of acute lymphoid disease is mitigated in the same model [33], and knocking down LYN by siRNA impairs survival of CML lymphoid blasts [34], indicating that the requirement of SFKs is greater in CML lymphoid transformation than in CP.

STAT5 is constitutively activated in CML. STAT5 downregulation mediated by siRNA in primary CML samples markedly impairs Ph + myeloid colony formation. Fetal liver hematopoietic progenitors from *STAT5a*$^{-/-}$:: *STAT5b*$^{-/-}$ mice retrovirally transduced with BCR-ABL1 failed to induce leukemia in recipient mice [35]. Conditional deletion of STAT5 in purified stem cells from p210$^{BCR/ABL}$ mice failed to initiate CML in mice [36]. Furthermore, the anti-apoptotic protein BCL-X, which is repressed by the transcription factor interferon consensus sequence-binding protein (ICSBP) [37], is transcriptionally activated by STAT5 [38].

Although the individual contribution of some BCR-ABL1 downstream pathways may appear negligible when evaluated individually, a cooperative interplay may be necessary for the full realization of the leukemogenic potential of BCR-ABL1. For instance, when *BCR-ABL1*-positive K562 cells were induced to express dominant negative forms of RAS, PI3K, or STAT5, marked apoptosis was observed in cells expressing two of the three dominant negative mutants in any combination [39].

RAC guanosine triphosphatases (GTPases) are activated in primary CML cells [40]. In a murine model of p210$^{BCR-ABL1}$-induced myeloproliferative disease, targeting of *RAC1* and *RAC2* genes, which encode the GTPases RAC1 and RAC2, markedly delayed myeloproliferation and abrogated the phosphorylation of the BCR-ABL1 downstream signaling molecules CrKL, JNK, ERK, and p38 [40]. This suggests that BCR-ABL1 signaling is highly dependent on RAC GTPases. This contention is supported by experiments using NSC23766, a specific RAC1/RAC2 inhibitor [41].

JUNB is a transcription factor that belongs to the activator protein 1 family. It functions as a tumor suppressor in myeloid cells [42]. JUNB exerts its tumor-suppressor activity by abrogating cell proliferation and survival through inhibition of the RAS downstream target JUN. Transgenic mice lacking the *JUNB* gene in the myeloid lineage (*JunB*$^{-/-}$Ubi-*JunB* mice) develop myeloproliferation that closely resembles CML; a fraction of these animals progress to BP [43, 44]. Granulocyte-macrophage colony-stimulating factor (GM-CSF)-mediated proliferation and survival of *JUNB*-deficient granulocyte/macrophage progenitors (GMPs) are associated with changes in anti-apoptotic proteins such as Bcl2 and Bclx and in cell cycle regulators p16ink4a and *c-Jun* [43].

Mice lacking the enzyme 12/15-lipoxygenase (12/15-LO) develop a myeloproliferative disorder with 100% penetrance that progresses to transplantable leukemia independent from ABL1 dysregulation [45]. Cells isolated from chronic-stage 12/15-LO-deficient mice (*Alox15*) exhibit increased PI3K/AKT activation as well as ICSBP, which results in decreased direct DNA binding, limiting the ability of ICSBP to repress BCL-2 gene transcription and promoting leukemic cell survival (Fig. 4.2) [45]. ICSBP is a negative regulator of granulocyte differentiation. *ICSBP*$^{-/-}$ and *ICSBP*$^{+/-}$ mice exhibit deregulated hematopoiesis manifested as a CML-like myeloproliferative disorder [46]. Forced expression of ICSBP inhibited *BCR-ABL1*-induced CML-like disease in vivo [47]. All the effects observed in *Alox15* mice were reversed upon treatment with a PI3K inhibitor. This suggests that 12/15-LO is an important suppressor of myeloproliferation [45]. Additionally, the arachidonate 5-lipoxygenase (5-LO) gene (Alox5) was identified as a critical regulator for CML LSC. BCR-ABL failed to transform bone marrow from Alox5-deficient mice, and both genetic or pharmacologic inhibition of Alox5 impaired the function of leukemic stem cells (LSCs) [48].

Progression to Advanced-Phase CML

The understanding of the mechanisms associated with the progression from CP to AP and BP has been dramatically hampered by the lack of animal models that faithfully recapitulate the process of CML transformation in vivo, which is almost invariably preceded by a protracted "chronic" myeloproliferative phase. However, several factors have been identified in samples from patients with AP CML as central to the process of transformation (Fig. 4.3). Genome-wide analysis of gene expression profiles may aid in characterizing gene candidates responsible for disease progression. Unfortunately, most studies have failed to provide clear-cut answers, likely because of differences in types of samples, array platforms, and/or statistical methodologies employed [49–54]. DNA microarray analysis comparing the gene expression in 91 cases of CML in all phases found striking similarities between gene expression in AP and BP. This suggests that CML might conform to a biphasic rather than a classic triphasic model of progression [54]. Genes potentially involved in AP/BP compared to CP included cytokines (IL3RA, SOCS2), alternative RAS pathways (Rras2), DNA-damage response genes, and genes involved in HSC self-renewal and interaction with the bone marrow stroma, such as those that encode effectors in the Wnt pathway (e.g., β-catenin) [54]. A genome-wide screening using single-nucleotide polymorphism (SNP) arrays identified only 0.47 copy number alterations per CML CP case (range 0–8), suggesting that *BCR-ABL1* is sufficient to induce CML. In contrast, a mean

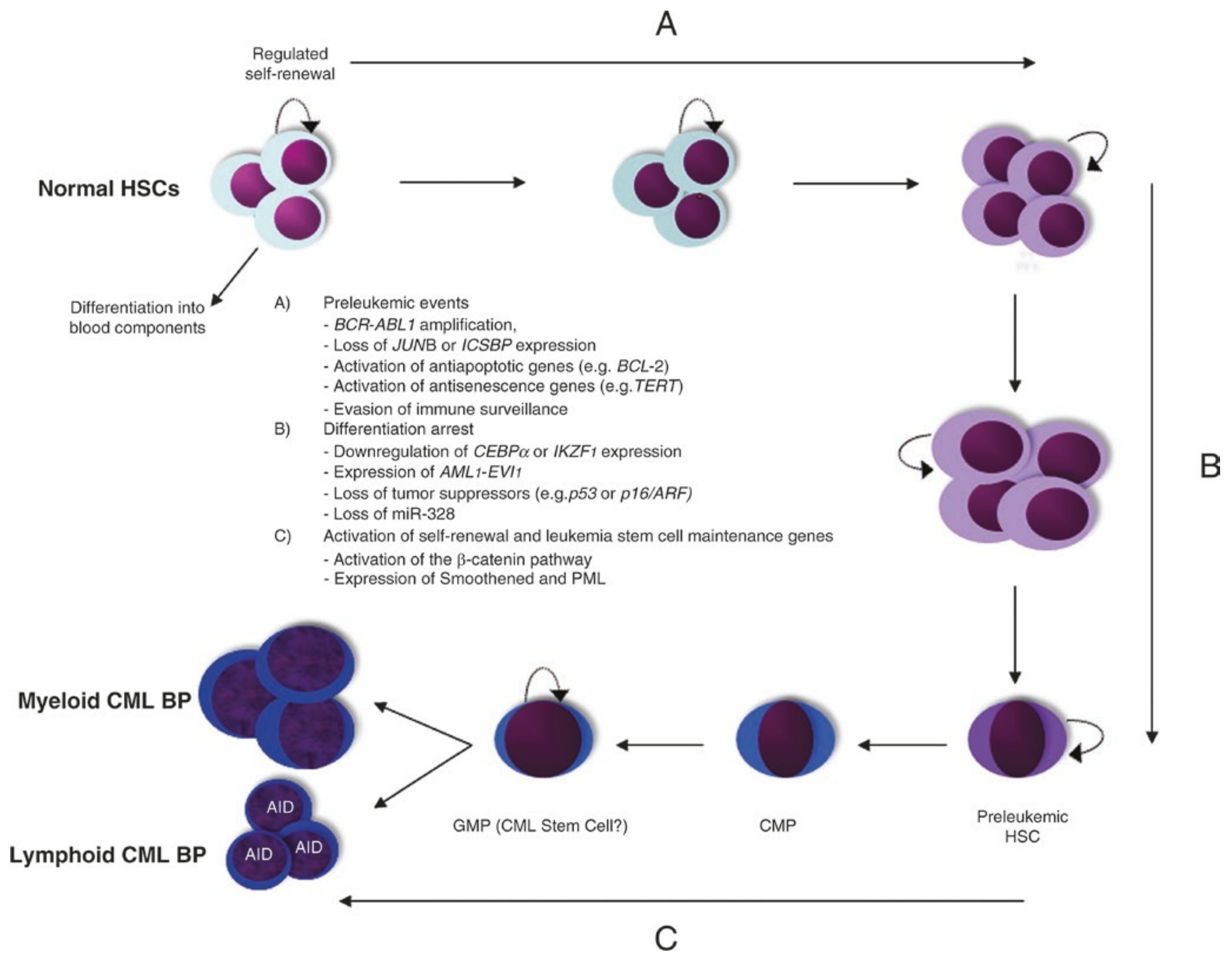

Fig. 4.3 Progression to blastic phase. In CML, hematopoietic stem cells (HSCs) accumulate genetic abnormalities that result in enhanced proliferation, survival advantage, cytokine independence, immune evasion, decreased apoptosis, and differentiation arrest. Granulocyte–monocyte progenitors (GMP) carrying such abnormalities are endowed with self-renewal potential, feed the leukemic pool during disease progression, and facilitate the emergence of clonal leukemic stem cells (LSCs) that drive the transition to blast-phase (BP) CML. Expression of activation-induced deaminase (AID) drives CML progression toward the lymphoid lineage

of 7.8 copy number alterations were detected per CML BP case (range 0–28), indicating that progression requires the acquisition of additional genomic alterations [55].

Blastic Phase Arises from Primitive CML Progenitors

In CML, CD34^{+}CD38^{-}Lin^{-} LSCs express high *BCR-ABL1* transcript levels [56]. LSCs acquire additional genetic and/or epigenetic abnormalities during the transition from CP to BP that confer resistance to apoptosis, extended replicative life span, and consequently a survival advantage [57, 58]. Mice genetically null for the *JUNB* [43] or *ICSBP* alleles develop a CML-like myeloproliferative disorder that, in the case of *JUNB*, arises from the HSC compartment and in some cases progresses to BP. JunB inactivation increases the proliferation of long-term repopulating HSCs in vivo without impairing their self-renewal potential [59]. Whether *BCR-ABL1* directly abrogates or cooperates with JunB expression to promote myeloproliferation during CML progression warrants further investigation, as does the determination of the cell of origin from which the BP clone arises. While transforming clones appear to arise from the HSC compartment in *JUNB*$^{-/-}$ mice, some reports suggest that the acquisition of self-renewal properties by a subset of committed progenitors may result in a CML-like phenotype that includes progression to BP in a mouse model in which the hMRP8p210$^{BCR\text{-}ABL1}$ transgene is expressed specifically by GMPs and their myelomonocytic progeny but not by HSCs [60]. These data suggest that BP may result from the progressive acquisition of genetic alterations within progenitors downstream of the

HSC that acquire self-renewal properties [60]. Importantly, GMPs have also been proposed as candidate LSCs in human CML BP [56].

CML BP Cells Overexpress BCR-ABL1

The continuous and unrestrained activity of BCR-ABL1 kinase is central not only for CML maintenance but also in the progression from CP to BP. *BCR-ABL1* mRNA and protein levels are consistently higher in BP than in CP [61, 62]. *BCR-ABL1* transcript levels may be 200-fold greater in CML CD34+ progenitors than in more differentiated *BCR-ABL1*-positive cells [63]. *BCR-ABL1* has been shown to promote clonogenicity [63, 64], growth factor independence [63], protection against apoptosis [64], cell motility [63], and disease latency in a dose-dependent manner [63]. The reason for the increment of *BCR-ABL1* transcript levels is not well understood, but this phenomenon is likely a consequence of the proliferative advantage resulting from high BCR-ABL1 kinase activity and therefore from selective pressure favoring the expansion of highly proliferative/poorly differentiated leukemic clones. Experimental evidence has demonstrated that levels of *BCR-ABL1* mRNA in the CD34+ GMPs were higher in CML BP than in CML CP [56]. *BCR-ABL1*-positive GMPs have been demonstrated to be expanded in CML BP [56]. Alternatively, it has been proposed that the BCR-ABL1 oncoprotein promotes the expression of *BCR-ABL1* transcripts and/or that *BCR-ABL1* transcript degradation may be selectively downregulated in CD34+ CML progenitors [65]. *BCR-ABL1*-independent mechanisms may also play an important role in CML progression. For instance, overexpression and/or activation of the SFKs HCK, LYN, and FYN has been linked to CML progression and imatinib resistance [66–69]. *BCR-ABL1* retrovirally transduced into bone marrow cells derived from *LYN*$^{-/-}$*HCK*$^{-/-}$*FGR*$^{-/-}$ mice efficiently induced CML but not B-ALL in recipient mice, which suggests that SFKs may play an important role in the pathogenesis of Ph + B-ALL and lymphoid CML BP [33, 70]. BCR-ABL1-independent activation of LYN was noted in patients with imatinib-resistant CML expressing unmutated *BCR-ABL1* [71]. Anti-SFK therapy may therefore be beneficial in advanced-phase CML [70].

Arrest of Differentiation

Progressive corruption of the differentiation program characterizes CML progression. BCR-ABL1 modulates the activity of transcription factors that regulate the expression of several differentiation-related genes (Fig. 4.3) [72, 73]. The *IKZF1* gene, which encodes the transcription factor Ikaros, is essential for lymphoid lineage specification. Ikaros isoforms lacking the DNA-binding domain act in a dominant negative fashion, preventing the generation of the earliest lymphoid progenitors and of mature lymphocytes [74]. SNP array analysis identified the presence of *IKZF1* deletions (typically monoallelic deletions of exons 3–6) in more than 80% of patients with Ph + ALL. Deletions were not present in CML CP cases but were found in 66–75% of CML CP cases transformed to lymphoid (but never to myeloid) CML BP [55, 75]. Downregulation of Ikaros expression causes partial block of B-cell maturation at the pro-B-cell stage in mice [76], and inactivation of Ikaros function blocks the development of B-lymphoid progenitors at the pre-B-cell stage [77]. *IKZF1* haploinsufficiency or the expression of dominant negative Ikaros isoforms may contribute to the pathogenesis of *BCR-ABL1*-positive lymphoid malignancies by inducing arrested B-cell maturation.

The transcription factor CCAAT/enhancer binding protein-α (CEBPα), a master regulator of myeloid differentiation [78], is expressed in normal bone marrow cells and in CML CP samples but not in CML BP [79]. Transplantation of *BCR-ABL1*-expressing CEBPα$^{-/-}$ fetal liver cells fails to induce myeloproliferative disease in mice. Rather, it induces an immature, lethal transplantable erythroleukemia. Transplantation of CEBPα-transduced cells consistently yielded a disease that closely resembles CML CP [80], suggesting that CEBPα downregulation abrogates cell commitment toward a myeloid cell fate. Differentiation arrest in myeloid CML BP is driven by BCR-ABL1/MAPK-induced activity of the poly(rC)-binding protein heterogeneous nuclear ribonucleoprotein E2 (hnRNP E2), which regulates the translation of specific mRNAs [79]. Expression of hnRNP E2 is low or undetectable in CML CP but is high in CML BP CD34+ bone marrow progenitors. Upon interaction with the 5′-untranslated region of *CEBPA* mRNA causes suppression of CEBPα [79]. MicroRNAs (miRNAs), small noncoding RNAs that have been shown to be important regulators of oncogene and tumor-suppressor gene expression in human cancer, can also inhibit the activity of RNA-binding proteins [81]. Indeed, miR-328 binds hnRNP E2, likely through its C-rich clusters in a seed sequence-independent manner, and as a consequence prevents the binding of hnRNP E2 to *CEBPA* mRNA, thus rescuing *CEBPA* mRNA translation and myeloid maturation. Not surprisingly, miR-328 expression is lost in CML BP in a BCR-ABL1 dose- and kinase-dependent manner through the MAPK-hnRNP E2 pathway. Restoration of miR-328 rescued differentiation and impaired the survival of *BCR-ABL1*-positive blast cells, partly by targeting the survival factor PIM [81].

Hybrid dominant negative transcription factors arising from chromosomal translocations such as *NUP98-HOXA9* [82] or *AML1-EVI1* [83] have been implicated in the pathogenesis of CML BP. NUP98-HOXA9 alters the balance between symmetric and asymmetric renewal division,

favoring the former and causing preferential growth of immature precursors, while *AML1-EVI1* has been shown to cause differentiation blockade, in both cases facilitating the transition to CML BP [84]. NUP98-HOXA9 induces expression of the RNA-binding protein Musashi2 (Msi2), which in turn represses differentiation factor Numb, known to be expressed at low levels in CML BP [85]. Msi2, normally highly expressed in HSCs and overexpressed in advanced CML, is associated with poor survival. In a mouse model, it cooperates with BCR-ABL1 to induce an aggressive leukemia [86].

Genomic Instability and DNA Repair

BCR-ABL1 expression has been associated with increased production of endogenous reactive oxygen species (ROS) that result in chronic oxidative DNA damage, double-strand breaks (DSBs) in S and G_2/M cell cycle phases, and mutagenesis [87]. DNA damage surveillance is faulty in CML owing to inhibited ataxia telangiectasia and RAD3-related (ATR) nuclear protein kinase signaling, which attenuates the activation of checkpoint kinase 1 (CHK1) and abrogates the intra-S-phase cell cycle checkpoint [88]. Nonhomologous end-joining and homologous recombination as well as nucleotide excision repair exhibit unfaithful repair of DSBs induced by ROS [87] and γ-irradiation [89] as a consequence of BCR-ABL1 kinase activity [87]. As a result, G/C-to A/T-transitions and G/C-to-T/A transversions in the coding regions of multiple genes (including the kinase domain of *BCR-ABL1*) have been demonstrated in CML cells but not in *BCR-ABL1*-negative cells [87, 90]. It has been shown that lymphoid (but not myeloid) CML BP cells express the B-cell-specific mutator enzyme activation-induced deaminase (AID), which contributes to genetic instability by hypermutation of tumor suppressor and DNA repair genes and to induction of mutations in the kinase domain of *BCR-ABL1* [91]. These results indicate that ROS generated by BCR-ABL1, in addition to aberrant regulation of DNA repair, contribute to a mutator phenotype in CML cells and lead to genomic instability that results in cytogenetic aberrations, point mutations, and consequently TKI resistance [90, 92]. Indeed, *BCR-ABL* has been shown to disrupt proteins involved in DNA DSB repair [93–95] and to cause cell cycle arrest in G_2/M in cells treated with DNA-damaging agents [96].

Chromosomal Abnormalities in CML

Approximately 80% of patients with CML develop additional nonrandom cytogenetic aberrations in Ph + cells, an occurrence known as "clonal evolution," which is a reflection of the genetic instability that characterizes the transition to advanced-phase CML [97]. In some reports, clonal evolution has been reported in advanced-phase CML at higher frequencies than *BCR-ABL1* mutations [98]. The most frequent cytogenetic abnormalities associated with clonal evolution are trisomy 8 (34%), isochromosome 17 (20%), and duplicate Ph chromosome (38%) [90, 99], which have been linked to c-*Myc* overexpression, loss of 17p (which results in *p53* loss), and *BCR-ABL1* overexpression, respectively [61, 100, 101]. Other cytogenetic abnormalities, such as trisomy 21, trisomy 17, and deletion 7, have been identified in less than 10% of cases of clonal evolution [102]. Also, 10–15% of patients with CML present with deletions within the derivative chromosome 9, which may lead to more rapid progression to BP [103]. In patients with 3q26.2 rearrangements (approximately 5%), the disease fails to respond to TKI treatment, exhibits a high rate of transformation to BP, and has a poor outcome [104, 105]. 3q26.2 gene rearrangements cause overexpression of a proto-oncogene, transcription factor EVI1 [106, 107]. Co-occurrence of RAS/receptor tyrosine kinase mutations contributes to leukemic transformation in myeloid malignancies [108]. Clonal evolution may therefore reflect a state of genetic instability that is frequently associated with advanced phases of CML and appears to play a pivotal role in CML progression. In the era of TKI therapy, single chromosomal change that includes trisomy 8, -Y, and an extra copy of Ph confers overall good response to therapy and favorable prognosis, while i(17)(q10), -7/7q (-7/del7q), and 3q26.2 rearrangements or concurrent emergence of more than two additional chromosomal abnormalities is associated with poor survival [109]. Cytogenetic abnormalities have also been detected in Ph-negative metaphases of 2–17% of patients with CML and have been occasionally linked with development of myelodysplasia or acute myeloid leukemia (AML) [110]. The SNP array findings that patients with CP CML harbored a mean 0.47 copy number alterations while those with BP CML harbored a mean 7.8 copy number alterations [55] further support the idea that multiple genomic aberrations accumulate during progression to BP CML.

Inactivation of Tumor-Suppressor Genes

The most frequently mutated tumor suppressor in human cancer is *p53*. In CML, *p53* can be found mutated during progression to BP in 25–30% of patients with myeloid CML BP. In addition to *p53*, exon 2 of the *INK4A/ARF* locus is deleted in 50% of cases of lymphoid CML BP [111], leading to loss of *p16* and *p14/ARF* expression, which regulates the G_1/S checkpoint by inhibiting the G_1-phase cyclin D-Cdk4/Cdk6 and by downregulating p53, respectively [100]. Deletion of the *P19ARF* gene in mice alters the nature of leukemia-initiating cells, rendering common lymphoid progenitors and precursor B-lymphocytes susceptible to transformation by BCR-ABL [112]. Because ARF enhances

p53 levels by interfering with the activity of MDM2, the principal negative regulator of p53, homozygous deletion at the *p16/ARF* locus represents a functional equivalent of *p53* mutation in myeloid CML BP. Clones of CML cells carrying such deletions are likely to be selected during CML progression [100]. Retroviral transduction of *BCR-ABL1* into *ARF*-null murine bone marrow cells rapidly generates polyclonal expansion of self-renewing pre-B cells [113]. Given the impact of p53 in disease progression, it is intuitive to think that alterations in the p53 pathway may also play a role in response to TKI therapy. Imatinib therapy causes p53 activation that is a direct consequence of BCR-ABL1 kinase inhibition [114]. By contrast, p53 inactivation has been shown to impair imatinib activity in vivo, suggesting that a mutated p53 pathway may contribute to imatinib resistance in advanced-phase CML [114].

The *RUNX* family of transcription factors has also been implicated in imatinib response in CML [115]. In mice transplanted with bone marrow cells retrovirally infected with *BCR-ABL1* and subsequently treated with imatinib to select for leukemic cells in which the proviral integration had affected genes modulating imatinib response, clonal outgrowth of cells carrying similar integration sites has been shown. Proviral integration near the *RUNX3* promoter induced expression of RUNX3, and *BCR-ABL1*-positive cell lines with stable or inducible expression of RUNX1 or RUNX3 were protected from apoptosis induced by imatinib treatment [115]. Imatinib therapy was also selected for *RUNX1*-expressing cells in vivo after infection of primary bone marrow cells with both *BCR-ABL1* and *RUNX1* [115], suggesting that *RUNX1* contributes to disease persistence.

Several miRNAs have been shown to act as tumor suppressors in CML and therefore they are subjected to a strong selection pressure. miRNA 203, which maps to 14q42, is frequently lost in lymphoid CML BP. Both *ABL1* and *BCR-ABL1* genes contain miR-203 target sequences. It has been shown that miR-203 is silenced through genetic (loss of one allele) and epigenetic (promoter CpG hypermethylation in the remaining allele) mechanisms, which suggests the existence of enormous selective pressure in CML cells to silence miR-203 in order to gain a proliferative advantage [116]. These data suggest that loss of miR-203 may play a critical role in CML progression. Decreased expression of other miRNAs such as miR-10a [117] and the miR-17-92 cluster [118] has also been associated with a gain in proliferative potential and possible transformation to CML BP. The RNA-binding protein Lin28 is highly expressed in CML BP, causing repression of miRNA let-7 and activation of let-7 targets, including signaling pathways important for CML proliferation [119].

PP2A functions as a tumor suppressor by antagonizing BCR-ABL1 [120]. BCR-ABL1 kinase inhibits PP2A by upregulating SET, a phosphoprotein that inhibits PP2A [120]. In turn, PP2A activates SHP1, which abrogates BCR-ABL1 phosphorylation and targets it for proteasomal degradation [120]. Both SET inhibition and PP2A activation represent potential therapeutic strategies. The PP2A activator fingolimod (FTY720) [121] induces apoptosis and impairs the clonogenic potential of the TKI-resistant 32D-p210(T315I)$^{BCR-ABL1}$ cell line and of primary bone marrow cells from patients with BP CML or Ph + B-ALL [122]. Fingolimod induced molecular remissions in severe combined immunodeficiency (SCID) mice transplanted with myeloid or lymphoid progenitors transformed with p210$^{BCR-ABL1}$ or p190$^{BCR-ABL1}$, respectively [122].

In summary, multiple lines of evidence suggest that the increased survival, proliferation, and differentiation arrest of CML BP cells relies upon the intimate cooperation of BCR-ABL1 with an array of genes deregulated during disease progression. A better understanding of these abnormalities and the generation of more faithful CML animal models will likely facilitate the development of more effective therapies for CML BP.

CML Stem Cells

The existence of LSCs was first demonstrated in AML, when upon transplantation into nonobese diabetic (NOD)–SCID mice, only the CD34 + CD38− population was capable of propagating AML in a new host [123]. These cells encompass <1% of the total number of AML cells. LSCs were then demonstrated in CML [124] and shown to contain two distinct populations of cells: a very small set consisting of highly quiescent diploid cells in the G_0 phase, and a larger one consisting of cells in $G_1/S/G_2/M$ [125]. While solid experimental data support the existence of a subset of leukemic cells capable of giving rise to new leukemic cells, thus promoting the proliferation of the malignant clone, the precise origin of such cells remains unclear. Ph + stem cells isolated from patients with CML treated with imatinib are able to repopulate immunocompromised mice, suggesting that TKIs are unable to eliminate quiescent CML stem cells [126]. Yet, approximately 40% of CML patients in the “Stop Imatinib Trial” (STIM) who achieved a complete molecular response were able to discontinue imatinib and maintain molecular remission [127], indicating either that CML stem cells are eliminated over time by the TKI or that minimal residual disease is eliminated through immune surveillance.

Quiescence and Persistence

One of the main features that sets *BCR-ABL1*-expressing LSCs apart from more committed leukemic progenitors is that LSCs are innately resistant to chemotherapeutic agents, radiation [128], and BCR-ABL1 TKIs [129–131]. Mathematical

models have shown that, when exposed to imatinib or other TKIs, CML cells decline in number in a biphasic pattern. During the initial stages of treatment, TKIs induce brisk and acute clearance of *BCR-ABL1* transcripts, likely a reflection of the elimination of the most differentiated CML cells. This is followed by a second slope of slower decline of *BCR-ABL1* transcripts, reflecting the targeting of more primitive CML progenitors, including CML LSCs, typically resulting in persistent residual disease [132, 133]. The factors underlying resistance of *BCR-ABL1*-positive LSCs to TKI therapy are not yet clearly defined. Factors invoked to explain this phenomenon include the following: (a) enhanced expression by CML stem cells of interleukin-3 (IL-3) and granulocyte colony-stimulating factor, which correlate with the primitive status of LSCs; (b) downregulation of the influx transporter human organic cation transporter-1 (hOCT1) and upregulation of the adenosine triphosphate-binding cassette transporter ABCB1 (MDR-1) and ABCG2, responsible for the influx and efflux of imatinib, respectively [65]; (c) increased expression of *BCR-ABL1* mRNA, protein, and kinase activity, which suggests that TKI-induced killing of CML LSCs requires doses much higher than those required to eliminate more mature *BCR-ABL1*-positive progenitors [134–136]; and (d) increased instability of the *BCR-ABL1* oncogene, which has been linked to increased levels of ROS and oxidative DNA damage, resulting in a higher rate of BCR-ABL1 kinase domain mutations in *BCR-ABL1*-positive LSCs even before exposure to therapy [137, 138]. There is therefore ample evidence that CML stem cells are resistant to the pro-apoptotic effects of TKIs, which may set the stage for CML relapse. However, other possibilities must be taken into account. For instance, in addition to imatinib, CML stem cells have proven highly resistant to the potent TKIs nilotinib and dasatinib [134–136]. In these cells, MAPK, PI3K, and STAT5 remain active despite BCR-ABL1 kinase inhibition [136]. Although CML LSCs express BCR-ABL [139], blockade of BCR-ABL activity by TKIs fails to eradicate CML stem cells [140, 141]. These observations suggest the interesting possibility that primitive quiescent CML stem cells may not depend on BCR-ABL1 kinase activation for survival. This would explain the TKIs' lack of activity against CML stem cells. An even more intriguing possibility is that TKI-induced BCR-ABL1 inhibition in CML stem cells might reverse the LSC phenotype to a "normal HSC" phenotype, thus calling into question the clinical importance of eradicating this subset of cells.

A potential way to eradicate LSCs is to stimulate their entry into the cell cycle [142, 143]. Interferon alpha (IFNα) has been shown to have this effect in quiescent CML stem cells. Upon binding to its receptor, IFNα induces STAT1 and STAT2 phosphorylation, which activates HSCs through the phosphorylation of AKT1 [142] and other factors involved in proliferation and by forming a complex with interferon regulator factor 9 (IRF9), which binds to the interferon-stimulated responsive element (ISRE) in genes whose transcription is regulated by IFNα. The negative regulator IRF2 competes with IRF9 for binding to ISRE, and its inactivation causes constitutive IFNα activity that causes HSCs to exit G_0 and enter an active cell cycle [142] that results in the depletion of the dormant HSC pool [144]. Therefore, while chronic activation of the IFNα pathway causes HSC depletion and compromises HSC function, acute INFα administration causes proliferation of dormant HSCs [142].

Stem Cell Maintenance Pathways

Several signaling pathways involved in embryonic development processes (tissue patterning, cell proliferation, differentiation) have been implicated in stem cell maintenance. These pathways have been shown to be involved in the pathogenesis of human cancer as they confer growth and/or survival advantage. The β-catenin pathway is key for HSC self-renewal in mice. Upon translocation to the nucleus, β-catenin interacts with its transcriptional coactivator lymphoid enhancer factor/T-cell factor (LEF/TCF) to modulate the transcription of genes such as *MYC* and *cyclin D1*. The transition from CP to BP in humans is characterized by a six- to tenfold expansion of GMPs rather than expansion of the HSC pool. Transfection of axin, a specific inhibitor of the β-catenin pathway, abrogates leukemic GMP replating and results in similar levels of β-catenin and LEF/TCF in HSCs in both normal controls and patients with CP or BP CML. Increased levels of β-catenin, LEF/TCF, and *BCR-ABL1* transcripts can be detected in GMPs isolated from patients in BP CML. The latter are endowed with increased self-renewal capabilities and stemness in vitro (as assessed by serial replating assays), compared with normal GMPs [56]. These data indicate the coexistence of two distinct self-renewing cell populations: one mainly consisting of GMPs that express high *BCR-ABL1* transcripts and enhanced nuclear β-catenin, which would be responsible for the transition to BP CML, and a second one involving *BCR-ABL1*-positive HSCs with a quiescent cell cycle, which would be responsible for the expansion and maintenance of the disease in CP. In this context, the role of the *BCR-ABL1* oncogene is not entirely clear, as this oncogene, unlike other leukemogenic fusion oncogenes such as *MLL-ENL* or *MOZ-TIF2*, can transform HSCs but is not sufficient to transform committed myeloid progenitors lacking inherent self-renewal capacity [145]. Additional pathways must therefore be implicated in LSC maintenance in CML.

The hedgehog (Hh) pathway is important during embryonic and postnatal development and in cancer [146]. Mutations in genes encoding proteins involved in the Hh pathway, such as *patched-1* (*PTCH1*) and *smoothened* (*SMO*), have been reported in several malignancies

[146–148]. Hh has been shown to play a role in primitive and adult hematopoiesis and is activated in LSCs [149–151]. Smo is a transmembrane receptor negatively regulated by the Hh receptor patched, which, in turn, is alleviated upon binding of the Hh proteins Shh, Ihh, or Dhh to patched [152]. In a murine model of CML, *BCR-ABL1*-expressing cells exhibited Smo upregulation, which was partially decreased upon treatment with imatinib or nilotinib [150]. Smo loss impairs HSC renewal, attenuates *BCR-ABL1*-induced CML, and depletes CML stem cells, likely through activation of Numb [152]. When $Smo^{-/-}$ cells transduced with *BCR-ABL1* were transplanted into irradiated mice, the number of LSCs was significantly lower than in control mice. Constitutively active Smo stimulates the propagation of CML stem cells and causes disease progression, supporting the important role of the Hh pathway in CML stem cells [152]. Pharmacological inhibition of Hh signaling with cyclopamine, which stabilizes Smo in its inactive form, abrogated the propagation of *BCR-ABL1*-positive cells and prolonged survival in a CML mouse model. These results, as well as recent data demonstrating the acquisition of *SMO* mutations that impair the binding of the Smo protein to Smo small-molecule inhibitors [153], highlight the importance of the Hh pathway in the pathogenesis of human cancer, and support the use of drugs that inhibit Hh signaling to eradicate LSCs in CML.

Blast cells obtained from patients with CML CP express high levels of the promyelocytic leukemia (PML) tumor-suppressor protein, which cannot be detected in more committed progenitors [154]. Genetic deletion of PML in mice or PML downregulation by arsenic trioxide resulted in increased cell cycling and ultimately exhaustion of HSCs that impaired hematopoietic reconstitution in recipient mice. CML leukemia-initiating cells (LICs) were generated by transduction of $p210^{BCR\text{-}ABL1}$ into $Pml^{-/-}$ HSCs. PML is therefore required for LIC mitotic quiescence and maintenance and this is accomplished through mTOR repression. Activation of mTOR with arsenic trioxide hampers HSC and LIC maintenance. Therefore, Hh inhibitors, mTOR activators, and IFNα are potential therapeutic strategies to eradicate LSCs in CML as they compromise LSC propagation, maintenance, and function. However, these may not be the only alternatives to effectively targeting LSCs, as other pathways appear to play a critical role in LSC homeostasis in CML. It has been shown that BCR-ABL1 kinase activates Akt signaling, which abrogates the forkhead O transcription factors (FOXO), resulting in proliferation and inhibition of apoptosis of CML cells [155]. By using a syngeneic transplantation system of immature bone marrow cells retrovirally transduced with *BCR-ABL1*, LICs have been found to be characterized by nuclear localization of FoxO3a and low Akt phosphorylation, both phenomena critically modulated by transforming growth factor-beta (TGF-β). In serial transplantation experiments utilizing LICs derived from $Foxo3a^{+/+}$ and $Foxo3a^{-/-}$ mice, genetic deficiency of Foxo3a remarkably limited the ability of LICs to induce a leukemic phenotype. In keeping with these results, TGF-β inhibitor therapy impaired the colony-forming ability of human $CD34^{+}CD38^{-}Lin^{-}$ CML cells in vitro [155], which suggests that the TGF-β–FOXO pathway has an essential role in the maintenance of CML LICs. Similarly, *ALOX5*, the arachidonate 5-lipoxygenase (5-LO) gene, has been identified as a critical regulator of LSCs in CML [156]. Alox5 deficiency renders BCR-ABL1 unable to induce CML in mice by impairing differentiation, cell division, and survival of LSCs but not normal HSCs. BCR-ABL suppresses Blk (encoding B-lymphoid kinase), which functions as a tumor suppressor in CML stem cells through an upstream regulator, Pax5, and a downstream effector, p27 [157].

Regulation of quiescence in LSC and insensitivity to TKIs is modulated by a pathway of peroxisome proliferator-activated receptor gamma (PPARγ) and its downstream targets STAT5 and HIF2α/CITED2, known to facilitate LSC dormancy [158]. In turn, activation of PPARγ by this antidiabetic glitazone drugs decreased STAT5 expression and reduced the LSC pool, causing stem cells to exit quiescence and eliminating disease when combined with TKIs. Additional pathways and transcriptional regulators implicated in CML stem cell maintenance include BCL-6 [159], MPL/JAK [160, 161], SIRT1 [162], Notch [163], Bmi1 [164], IL2/CD25 signaling [165], and autophagy [166]. Interactions between LSCs and bone marrow microenvironment may also influence the success of CML therapy [167, 168], and approaches targeting LSC-niche interactions have been shown to be effective in preclinical model systems [169, 170]. Additionally, the hypoxic nature of the marrow niche has been proposed to offer LSC maintenance and protection from TKIs [171].

BCR-ABL1 Kinase Domain Mutations and TKI Resistance

CML may fail to respond or may lose its response to TKI therapy through a series of BCR-ABL1-dependent or -independent mechanisms of resistance including TKI compliance and bioavailability, pharmacodynamics, genetic changes, *BCR-ABL1* kinase domain mutations, or BCR-ABL1 overexpression. The most frequent and clinically relevant mechanism of resistance to TKI therapy is the acquisition of mutations within the kinase domain of *BCR-ABL1* (Fig. 4.4). Mutations have been reported at frequencies ranging from 40 to 90% among patients with imatinib-resistant CML [172–177]. Kinases are plastic multimodular structures that oscillate between an active and an inactive conformation. Different TKIs bind different conformations of the BCR-ABL1 kinase. For instance, imatinib and

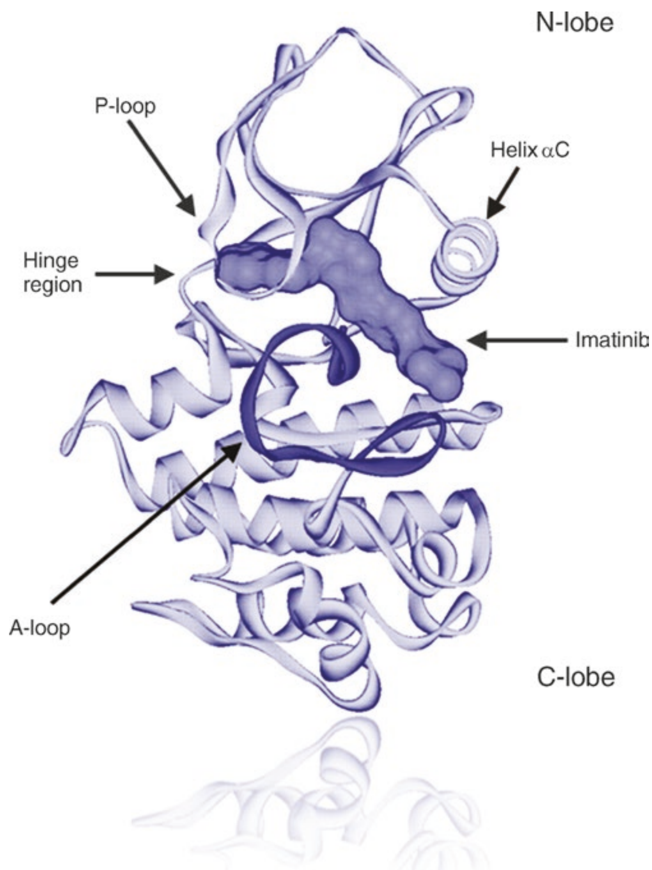

Fig. 4.4 3D structure of the ABL1 kinase domain in complex with imatinib. The ATP-binding site in the ABL1 kinase domain is located between the activation loop (A-loop) and the phosphate-binding loop (P-loop). The A-loop controls the ABL1 catalytic activity by switching between the active and the inactive conformations of the kinase. Imatinib inserts its pyridinyl group underneath the helix αC in the NH2-terminal lobe of ABL1 kinase, displacing ATP and freezing the kinase in its inactive conformation. Mutations mapping at the P-loop, the A-loop, and imatinib contact sites are the most common causes of acquired resistance to imatinib therapy

nilotinib bind the inactive conformation of ABL1 kinase, in which the highly conserved Asp-Phe-Gly (DFG) residues are swung out of the catalytic cleft ("DFG-out") [178, 179]. Imatinib extends deeply into the catalytic domain, and its pyridinyl group locates underneath the αC helix in the NH_2-terminal lobe of ABL1 kinase [178]. On the other hand, X-ray crystallography [180] and NMR spectroscopy [181] have shown that dasatinib binds the active ("DFG-in") conformation of ABL1.

Over 100 different point mutations encoding single-amino acid substitutions within the kinase domain of *BCR-ABL1* have been detected in patients with imatinib-resistant CML [20, 177, 178, 182, 183], and others have been generated in vitro by random mutagenesis of *BCR-ABL1* [182, 184]. Imatinib-resistant mutations tend to cluster at specific regions within ABL1 kinase. One such region, the P-loop region (residues 248–255) of the kinase domain, serves as a docking site for phosphate moieties of ATP [185–187]. While some studies linked P-loop mutations to a poorer clinical outcome [188, 189], others have not confirmed this observation [190]. In fact, not all P-loop mutations have the same transformation potency or sensitivity to different TKIs. Second-generation TKIs such as dasatinib, bosutinib, and, to a lesser extent, nilotinib are active against some P-loop mutations (Table 4.1). Other areas within ABL1 kinase frequently affected by mutations include the activation (A) loop (residues 381–402), whose mutations prevent the kinase from adopting the inactive conformation to which imatinib binds, and the catalytic (C) domain (residues 350–363). Particularly worrisome are those mutations that affect ATP-contact sites within the ATP-binding region (e.g., T315I, F317L, V299L). Selected ATP-binding loop mutations such as Y253F increase intrinsic BCR-ABL kinase activity [191, 192]. The gatekeeper residue Thr315 sits at the periphery of the nucleotide-binding site of ABL1 and forms an H-bond with imatinib and dasatinib [183]. Mutation of Thr315 to isoleucine (T315I) disrupts this H-bond interaction, which, in addition to the steric hindrance imposed by the isoleucine side chain and the stabilization of the kinase in the active conformation, impairs imatinib binding, causes complete insensitivity to this compound as well as to second-generation TKIs, and promotes malignant transformation [6, 174, 179, 193–197]. T315I mutation has been reported in approximately 15% of patients after failure of imatinib therapy [198]. For patients with a gatekeeper T315 mutation, the only TKI approved for clinical use is ponatinib [199]. Ponatinib is a type II inhibitor that avoids T315 by inclusion of a rigid triple-carbon bond. Ponatinib at higher concentrations is capable of inhibiting other BCR-ABL mutations (such as E225V), and these levels are clinically achievable, accounting for higher response rates to ponatinib in CML patients with multiple mutations [200]. The F317L and V299L mutations almost invariably arise during dasatinib therapy but retain sensitivity against nilotinib [201]. Mutations occurring at a subset of residues (Q253, Y253, E255, T315, E459, and F486) are more frequently detected in patients with advanced-phase CML [202].

Despite the wide variety of *BCR-ABL1* point mutations, most mutants are rare, with mutations involving residues Gly250, Tyr253, Glu255, Thr315, Met351, and Phe359 accounting for 60–70% of all mutations [203]. The presence of T315-inclusive compound mutations, i.e., two or more mutations within the same BCR-ABL molecule, confers resistance to all currently available TKIs [204] and has been found in patients in whom ponatinib failed [200, 205]. This is not the case for non-T315I compound mutations, which are variably sensitive to several TKIs. Over 60 different BCR-ABL1 compound mutations have been reported in association with sequential TKI use and resistance [206–208]. Clones with the E225K/T315 compound mutation

Table 4.1 Sensitivity of BCR-ABL1 kinase domain mutations to imatinib and the second-generation tyrosine kinase inhibitors nilotinib, dasatinib, and ponatinib

	$BCR\text{-}ABL_1$ isoform	Imatinib (nM)	Nilotinib (nM)	Dasatinib (nM)	Ponatinib (nM) [199]
	Native	260	13	0.8	0.5
P-loop	$M_{244}V$	2000	38	1.3	2.2
	$G_{250}E$	1350	48	1.8	4.1
	$Q_{252}H$	1325	70	3.4	2.2
	$Y_{253}H$	>6400	450	1.3	6.2
	$Y_{253}F$	3475	125	1.4	2.8
	$E_{255}K$	5200	200	5.6	14
	$E_{255}V$	>6400	430	11	36
	$V_{299}L$	540	NA	18	NT
ATP-binding site	$F_{311}L$	480	23	1.3	NT
	$T_{315}I$	>6400	>2000	>200	11
	$T_{315}A$	971	61	125	1.6
	$F_{317}L$	1050	50	7.4	1.1
	$F_{317}V$	350	NA	53	10
Catalytic domain	$M_{351}T$	880	15	1.1	1.5
	$E_{355}G$	2300	NA	1.8	NT
	$F_{359}V$	1825	175	2.2	10
	$V_{379}I$	1630	51	0.8	NT
A-loop	$L_{387}M$	1000	49	2	NT
	$H_{396}R$	1750	41	1.3	NT
	$H_{396}P$	850	41	0.6	1.1

NA not applicable, *NT* not tested

were shown in vitro to produce paracrine factor IL-3, which promotes survival of bystander non-mutated cells through the MEK/ERK and JAK2/STAT5 pathways [209]. Novel allosteric BCR-ABL inhibitors binding in non-ATP pocket sites have entered clinical trials and have been shown in preclinical studies to restore the sensitivity to conventional TKIs of the T315I mutant [210]. Whether such inhibitors can block compound mutations remains to be determined.

It is worth emphasizing that different mutations are endowed with different transforming capabilities that are not tightly related to their kinase activity. In pre-B-cell transformation assays, T315I (which has weaker kinase activity than $p210^{BCR\text{-}ABL1}$) and E255K consistently showed a 10–20% increase in oncogenic potency relative to that of $p210^{BCR\text{-}ABL1}$; whereas the P-loop mutants Y253F and E255V had potencies similar to those of $p210^{BCR\text{-}ABL1}$ and Y253H, T315A, F317L, and M351T were markedly weaker [192]. Relative to unmutated *BCR-ABL1*, Y253F and E255K mutants have higher transformation potency, whereas M351T and H396P mutants are less potent. The kinase activity of E255K, H396P, and T315I did not correlate with transforming potency. Analysis of the phosphotyrosine proteome by mass spectroscopy confirmed the presence of different phosphorylation signatures among the different mutants, confirming that different mutations determine substrate specificity leading to activation of different downstream pathways [191]. Importantly, some patients with CML tend to accumulate more than one BCR-ABL1 mutation, frequently within the same *BCR-ABL1* allele, when they experience sequential TKI therapy failure, and this accumulation of mutations was associated with greater oncogenic potency than each individual mutation [206].

BCR-ABL1 kinase-independent mechanisms of resistance are thought to involve activation of alternative signaling pathways and might involve multiple mechanisms. For example, activation of pSTAT3, STAT5, MAPK, JAK2, LYN, SYK, PI3K, and XPO1/CRM1 has been implicated in kinase-independent resistance to TKIs [68, 211–219]. Other mechanisms of resistance include impaired inhibitor influx or increased drug efflux, such as cation transporter OCT1 for imatinib resistance [220, 221] and MDR1 for nilotinib resistance [222].

Concluding Remarks

The central role of *BCR-ABL1* as the causative agent in the pathogenesis of CML is firmly established. Cells expressing *BCR-ABL1* acquire growth factor independence and exhibit deregulated cell proliferation and increased resistance to apoptosis. These capabilities are acquired and sustained by a complex network of signals that emanate from the constitutive kinase activity of BCR-ABL1. Such signals result initially in unbridled myeloproliferation of

mature myeloid elements; over time CML cells exhibit a marked loss of differentiation and growth arrest at the very early steps of myeloid maturation, which, in the absence of appropriate therapy, results in the transformation to BP. The initial steps of the pathogenesis of CML are directly choreographed by BCR-ABL1. Its activity during transformation, while necessary, is no longer sufficient. The mechanisms that govern the transformation process are not well understood, but research advances in this area have unveiled the previously unknown involvement of a series of transcription factors, miRNAs, and tumor suppressors in this process. Accordingly, blocking the constitutive activation of BCR-ABL1 kinase with TKIs such as imatinib results in high rates of response and a dramatic prolongation of survival among patients with CML CP. However, this strategy is not durably effective in patients with CML BP. It is therefore important to continue unraveling the intimate molecular mechanisms of transformation to better devise therapeutic strategies for patients with advanced-phase CML. In addition, while current TKI therapy for CML CP is highly effective, it does not fully eradicate the leukemic clones in most patients. The persistence of circulating and bone marrow-based CML cells is likely a consequence of the lack of sensitivity of primitive progenitors and CML stem cells to TKIs. A better understanding of the mechanisms that regulate CML stem cell homeostasis would facilitate the development of molecularly curative strategies for patients with CML.

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5 Diagnosis and Treatment of Chronic Myeloid Leukemia

Charles A. Schiffer

Introduction

The original recognition of leukemia in the nineteenth century and the story of our progressive understanding of the biology and the development of treatment of chronic myeloid leukemia (CML) have been well reviewed in recent years [1–3]. Today, the diagnosis of CML usually presents few problems. In contrast, planning a therapeutic strategy for a patient who presents in chronic phase and monitoring a patient who starts treatment with a tyrosine kinase inhibitor (TKI) present a number of challenges. The same is true for a patient in chronic phase whose disease proves resistant to initial treatment with a TKI. Even more difficult may be the issue of how best to treat a patient presenting in or progressing to an advanced phase of CML. In this chapter, we review some of the essentials of diagnosis of CML with the main focus on the results of available treatment options and guidance on therapeutic strategy.

Diagnosis

Definition, Diagnostic Criteria, and Differential Diagnosis

CML is a clonal myeloproliferative expansion of transformed primitive hematopoietic progenitor cells involving myeloid, monocytic, erythroid, megakaryocytic, B-lymphoid, and occasionally T-lymphoid lineages [4]. Since 1960 when Nowell and Hungerford [5] described the specific karyotypic abnormality, a G group chromosomal abnormality that came to be known as the Philadelphia (Ph[1] or Ph) chromosome, there has been rapid progress in our understanding of the pathogenesis of the leukemia, providing us with the means to easily diagnose and monitor the disease. Subsequent studies further characterized the Ph to be the result of a balanced translocation between chromosomes 9 and 22 (t(9;22)(q34;q11)) [6]. A series of subsequent studies demonstrated that this resulted in a fusion gene involving the *BCR* (breakpoint cluster region) gene from chromosome 22 and the Abelson cellular oncogene, *ABL* from chromosome 9, which produces a chimeric protein which is responsible for the constitutive proliferation of myeloid cells [9]. Mice transfected with the *BCR-ABL1* fusion gene develop a myeloproliferative disorder resembling human CML or other Ph1 acute leukemias [1, 7–9].

The detection of the BCR-ABL1 fusion gene is the pathognomonic feature of almost all cases of CML. A few conditions demonstrate overlapping clinical features, the most common being a “leukemoid reaction” which occurs usually in response to severe infection. In contrast, however, the presence of splenomegaly and a low leukocyte alkaline phosphatase score (a test which is no longer performed routinely) suggest a diagnosis of CML. The presence of the characteristic Ph chromosome will allow the distinction of CML from disorders such as primary proliferative polycythemia, idiopathic myelofibrosis, and primary thrombocythemia, which can occasionally have a somewhat overlapping clinical presentation. The identification of *BCR-ABL1* in a peripheral blood sample by reverse transcriptase-polymerase chain reaction (RT-PCR) techniques or by fluorescence in situ hybridization (FISH) will give the definitive answer, though approximately 5% of patients with a blood picture resembling CML are negative for the Ph chromosome by cytogenetics [10, 11].

Among such Ph-negative patients, there is a preponderance of males and older patients, with lower leukocyte counts and thrombocytopenia being more typical of this subgroup. Of those patients who lack a Ph chromosome, about half are also *BCR-ABL1* negative; they are sometimes designated as “atypical CML” and their prognosis is poorer than that of patients with *BCR-ABL1*-positive leukemia

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[10]. The other half have cryptic *BCR-ABL1* fusion gene on a normal-appearing chromosome 22 and such patients are usually designated Ph-negative, *BCR-ABL1*-positive CML. Their clinical features and response to treatment with TKIs differ little, if at all, from those of patients with a Ph chromosome. Specific mention should also be made of those patients who appear to have primary thrombocythemia but with a Ph chromosome and a *BCR-ABL1* gene. Such patients should be considered to have CML and should be managed as if they had classic CML. It is therefore recommended that all patients with apparent primary thrombocytosis should be tested for the Ph translocation and/or *BCR-ABL1* by RT-PCR.

Evaluation of a Suspected Case of CML

The specifics of the investigation of a newly presenting patient with CML are detailed in Table 5.1. In the presenting history, it is helpful to ask about certain features, such as the presence of night sweats or bone pain, as they may indicate transforming disease. Symptoms suggestive of hyperviscosity such as headaches, confusion, and visual disturbances are important to identify, but occur infrequently even in patients with very elevated WBC. It may be helpful to determine exposure to potential mutagens, especially high levels of ionizing irradiation, although it is very unusual for CML to develop as a "secondary" leukemia. There was a transient increase in the incidence of CML in Japanese survivors in the first decade after the atomic bomb exposure [12–14], but it is not clear that irradiation in the doses used for therapy of other cancers results in CML. Examination should particularly focus on retinal examination and lymph node areas, and include documenting the size of the spleen and liver. It is important to reassure the patient and family that the disease is not inherited, and to establish whether the patient has any siblings and hence potential for allografting.

Table 5.1 Investigations to be performed in suspected cases of CML

• CBC with differential and review of blood film
• Biochemistry screen including uric acid
• Bone marrow aspirate and trephine biopsy for:
Morphology (assess cellularity and degree of fibrosis on biopsy) • Cytogenetics (fluorescent in situ hybridization [FISH] if metaphase cultures fail) • Sample for immunophenotyping (process only if blast crisis is evident morphologically; not necessary if typical chronic phase) • Samples stored for research purposes if appropriate locally or for mailing to research group • RT-PCR if not available from peripheral blood (needed to define breakpoint for future monitoring)
Consider CMV serology and HLA type patient and siblings if allograft is being considered

A complete blood count and film review are critical in establishing the prognostic score (discussed later), and the number of blasts, basophils, and eosinophils should be calculated for this purpose. Immunophenotyping is only relevant to classify blast crisis. The trephine biopsy should be assessed for cellularity and the degree of fibrosis. Complete karyotypic analysis is necessary to assess for the presence of additional cytogenetic abnormalities in the Ph clone since FISH and RT-PCR will not detect abnormalities on chromosomes other than 9 and 22. It should be noted that all of these studies, save the assessment of marrow fibrosis, can be done on peripheral blood, since, because of the presence of circulating immature elements, chromosome analysis can generally be done successfully. Hence, some clinicians forego bone marrow evaluation in patients with typical, early-stage disease.

Clinical Presentation and Phases of Disease

Savage et al. [15] described a series of 430 consecutive cases presenting to one center for consideration of allogeneic transplant and these data are the first description of the presenting features of such a large group of CML patients in the modern era (Table 5.2). Approximately 20% of the patients were diagnosed when a blood sample was taken for other reasons. It is likely a higher fraction of people in developed countries are currently diagnosed "incidentally" given the increased use of "routine" screening blood tests. Some cases were diagnosed during pregnancy, while donating blood, or undergoing routine surgery.

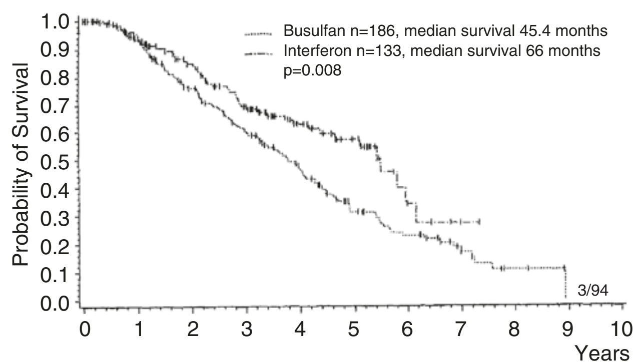
Of those presenting with symptoms, the 10 most commonly recorded are shown in Table 5.2. In retrospect, patients may describe fatigue, weakness, or a sense of fullness in the left upper quadrant with early satiety after meals. Other symptoms including visual disturbance, weakness, arthralgia, cough, malaise, dizziness, nausea/vomiting, ankle edema, priapism, and mental changes occurred in less than 5% of the cases. Thrombocytosis with a count above 1×10^{12}/L was found in 25% of cases, although there did not appear to be any correlation between abnormal bleeding and the level of thrombocytosis. Splenomegaly and purpura were the most common physical signs at presentation, at 75% and 16%, respectively.

Ninety-three percent of the patients presented in chronic phase, i.e., with fewer than 5% blasts in the bone marrow. It is sometimes difficult, particularly if the marrow or blood smear staining is not done well, to confuse younger myeloid elements with true blasts, and it is not unusual to have the appearance of a somewhat higher percentage of blasts at presentation, although it eventually becomes clear that the

Table 5.2 Clinical presentation of 430 patients referred to the Hammersmith Hospital for consideration of transplantation from 1981 [15]

• 80% of the patients were symptomatic at the time of presentation, although most symptoms were mild to moderate in severity	
• 20% of the patients had the diagnosis of CML made incidentally on routine CBC	
• 93% of the patients presented with chronic-phase disease	
• Thrombosis and leukostasis were rare even with very high platelet/white blood cell counts	
Median CBC values (range):	
WBC	174 (5.0–850.0) × 10^9/L
Hemoglobin	10.3 (4.9–16.6) g/dL
Platelets	430 (17–3182) × 10^9/L
• 19% of the patients presented with a WBC >350 × 10^9/L	
• 25% of the patients presented with platelets >1 × 10^{12}/L	
Most common symptoms at presentation	
Fatigue and lethargy	33%
Weight loss	20%
Abdominal mass or fullness	15%
Bone pain	7%
Headache	6%
Bleeding	21%
Splenic discomfort	19%
Sweats	15%
Infection	6%
Dyspnea	~5%
Most common findings on physical examination	
Spleen palpable	75%
1–10 cm	37%
>10 cm	39%
Purpura	16%
Palpable liver	2%

The median age of 34 years was somewhat younger than CML in general because these were patients referred for transplantation



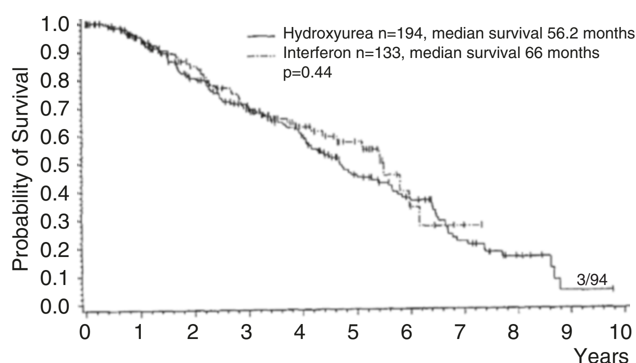


Fig. 5.1 These survival curves demonstrate the inexorable rate of death due to blastic transformation in patients not undergoing allogeneic transplantation in the pre-TKI era. Reprinted with permission [43]

Table 5.3 Criteria used in defining phases of disease in CML

WHO criteria for accelerated phase 2016 [17]
• >10–19% blasts in blood or marrow
Persistent or increasing WBC (>10 × 10^9/L), unresponsive to therapy
• >20% basophils in blood
• Thrombocytopenia (<100 × 10^9/L) unrelated to therapy
• Persistent thrombocytosis (>1000 × 10^9/L) unresponsive to therapy

patient was truly in chronic phase after observing the response to treatment.

Before the introduction of tyrosine kinase inhibitors (TKI), the duration of chronic phase was usually between 3 and 8 years (median 4–5 years in most series), although exceptions occurred, with some patients evolving rapidly to blast crisis, whereas others did not progress for 15 or more years (Fig. 5.1). In the past, the disease inevitably progressed from this "benign" chronic stage to the accelerated phase and ultimately to a fatal blast crisis. The situation is now very different. Patients who respond to treatment with a TKI and achieve a complete cytogenetic response (CCyR) may maintain this response for many years and possibly indefinitely, provided that they continue to take a TKI on a regular basis. Thus, the progression from chronic phase to advanced phase that seemed in the past to be inevitable now appears to be preventable.

Accelerated Phase

The definition of *accelerated phase* is vague, and different criteria have been used in the past by different groups [16]. A recent definition proposed by the World Health Organization [17] is summarized in Table 5.3, with anemia, increasing basophils or eosinophils, thrombocytopenia (or occasionally thrombocytosis), or increasing proportion of blasts being the most common findings. Clinical features suggesting "acceleration" supplement these laboratory findings and commonly include fever, night sweats, weight loss, bone pain, increasing splenomegaly despite therapy, and development of extramedullary disease (chloromas).

Failure to achieve a hematologic response to initial therapy with a TKI, hematological, cytogenetic, or molecular resistance to two sequential TKIs, or the development of two mutations in BCR-ABL1 during TKI therapy, is also a worrisome feature, in the absence of the findings listed above.

Blast Phase

The definition of *blastic transformation* (also referred to as blast phase or blast crisis) is based on the presence of more than 20% blasts in the peripheral blood or bone marrow, or the demonstration of extramedullary infiltration by blast cells. In two-thirds of the cases, the blasts are myeloid with one-third of B-lymphoid lineage. The blasts are often undifferentiated morphologically and immunophenotyping is therefore recommended in all cases. Transformation to lymphoid blast crisis can occur suddenly, sometimes even early in the course of chronic phase, and carries a marginally better prognosis than myeloid transformation. Both are usually fatal despite intensive treatment and have a median survival from diagnosis of blast crisis of only 3–6 months, unless remission can be achieved and followed by allogeneic transplantation.

It can be difficult to distinguish patients presenting initially with Ph-positive acute myeloid leukemia (AML) or acute lymphoblastic leukemia (ALL) from those with blast crisis of CML. For patients with Ph + ALL, the presence of significant splenomegaly is more in keeping with preexisting, undiagnosed CML, whereas the presence of p190$^{BCR\text{-}ABL1}$ suggests de novo ALL while p210$^{BCR\text{-}ABL1}$ might suggest preexisting CML. It is possible, in both children and adults with CML, to develop blast transformation with mixed lineages, i.e., both lymphoid and myeloid surface markers detectable on the same cells [18] or distinct subpopulations of blasts with either lymphoid or myeloid characteristics [19]. T-lymphoid blast transformation is rare, but there are several cases showing both the *BCR-ABL1* fusion gene and T-cell receptor (TCR) gene rearrangements [20].

Cytogenetic and molecular changes are well recognized in 50–80% of the patients during transformation to accelerated or blast phase. So-called minor cytogenetic changes include monosomies of chromosomes 7 and 17, loss of the Y chromosome, and trisomies of chromosomes 17 and 21 [21]. Major cytogenetic changes, which suggest a more aggressive clinical course, include a double-Ph chromosome, trisomy 8, isochromosome i(17q), trisomy 19, and translocations of chromosome 3 with chromosome 21, t(3;21)(q26;q22) [22–24]. Alterations of the p53 gene on the long arm of chromosome 17 by deletion, rearrangement, or mutation, occurring predominantly with myeloid blast crisis, have been identified in up to 30% of CML patients entering the blast phase [25, 26]. Even before clinical manifestations, it is sometimes possible to detect these cytogenetic changes in the bone marrow, extramedullary masses, or splenectomy specimens [27].

Clinical signs of blastic transformation may be due to the rapid increase in blasts in the peripheral blood. The most significant areas compromised are the cerebral and respiratory circulations, resulting in multifocal bleeding, dyspnea, and hypoxemia [28]. Tumors due to the deposition of blast cells, known as chloromas or granulocytic sarcomas, may be visible before the detection of blasts in the peripheral blood. It is important to distinguish such tumors from undifferentiated carcinomas and diffuse large-cell non-Hodgkin's lymphoma, which may require immunohistochemical staining. Commonly, the tumors are detected in lymph nodes, cutaneous tissue, or as lucent bone deposits on X-ray. Meningeal deposition may result in cord compression or a variety of neurologic symptoms [29].

Prognostic Scores

Prognostic models aim to categorize patients into different risk groups at diagnosis. These scores all require examination of the first blood film made on the newly diagnosed patient, and the subsequent loss of this blood film is the most common reason for an incomplete score. Prognostic scores have traditionally been utilized in the context of analyzing large clinical trials and thus they should be applied to the individual patient with some caution. Small variations in the parameters described can make a significant difference to the final score; accurate determination of these blood values is therefore crucial. Older scores were proposed by Tura et al. [30], and subsequently in 1982 by Cervantes and Rozman [31]. More contemporary systems are described in Table 5.4. It should be noted that the outcomes for the Sokal and Hasford scores evaluated patients in the pre-TKI era whereas the EUTOS score included 2060 patients treated with imatinib between 2002 and 2006.

Gratwohl et al. [35] devised a simple scoring system based on five main factors following analysis of 3142 patients who had undergone allogeneic stem cell transplantation for CML between 1989 and 1997. The combined score for these factors (donor/recipient histocompatibility, stage of disease, patient and donor age and sex, and time from diagnosis) in an individual patient predicted the probability of survival after allografting and was helpful in the counseling of patients. Although many of these factors are probably still relevant, it is difficult to use this formula with precision now, given the major improvements in transplant methodology which have decreased transplant-related mortality, as well as possible improved outlook in patients treated predominantly with TKIs.

Table 5.4 Prognostic scores commonly used in CML

Sokal score (*1984*) [32]
• Criteria required at presentation (prior to treatment): 1. Age 2. Spleen size (cm below costal margin measured clinically with a tape) 3. Platelet count prior to any treatment 4. Blast percentage in peripheral blood (preferably 500 cells counted, but at least 200)
• Formula: Exp[0.0116(age—43.4) + 0.0345(spleen—7.51) + 0.188 (platelets/700) 2—0.563) + 0.0887 (% blasts—2.1)] Good prognosis <0.8 Moderate prognosis 0.8–1.2 Poor prognosis >1.2
Hasford (*Euro*) *score* (*1998*) [33]
• Data analyzed on 1573 patients
• Criteria required at presentation (prior to treatment): Same as Sokal score with the addition of: – Eosinophil percentage in peripheral blood (same no. of cells counted) – Basophil percentage in peripheral blood (same no. of cells counted)
• Formula: (0.6666 × age [0 when <50 years; otherwise 1]) +0.0420 × spleen size [cm from costal margin] +1.0956 × platelet count [0 when platelets <1500; otherwise 1] +0.0584 × blasts [%] +0.0413 × eosinophils [%] +0.2039 × basophils [0 when basophils <3%; otherwise 1] multiplied by 1000 Low risk ≤780—median survival 100 months Intermediate risk >780 < 1480—median survival 69 months High risk >1480—median survival 45 months
EUTOS score [34]
• Formula: (7 × basophil [%]) + (4 × spleen [cm]) Low risk <87 (79% of patients) High risk ≥87 (21% 0f patients)

An online calculator for the most commonly used scores is available at http://bloodref.com/myeloid/cml/sokal-hasford

Treatment

The introduction of the tyrosine kinase inhibitors in 1998 fundamentally changed the treatment of CML. Until that time it has been widely accepted that an allogeneic stem cell transplant, if the patient had a suitable donor and if the procedure was successful, could cure CML, whereas other therapies were essentially palliative and would modestly prolong life, if at all. Early studies with imatinib mesylate, the original TKI, showed that it could reduce Ph-negative hematopoiesis in patients shown previously to be resistant to interferon-alpha [36], and subsequent studies demonstrated that it had the capacity to induce durable CCyR in 60% or more of the patients presenting in chronic phase. It is now widely accepted that CML presenting in chronic phase should be treated initially with a TKI. The choice of which TKI to use is still a matter of discussion, as described below. Treatment decisions for patients presenting in advanced phases of CML are more difficult.

Immediate Management of Newly Diagnosed Patients

The immediate management of a newly presenting patient with possible CML involves the initial history taking and investigations described in the previous sections. The next step is to control any immediate life-threatening complications such as leukostasis, hemorrhage, or infection, before appropriate antileukemic chemotherapy. Tumor lysis syndrome is relatively rare in patients in chronic phase, but nonetheless, it is prudent to commence allopurinol 300 mg daily and encourage plentiful oral fluid intake. Once the leukocyte count has been reduced below 30×10^9/L, allopurinol should no longer be required unless the patient has a history of gout or continues to be hyperuricemic. On some occasions it may be necessary to reduce the leukocyte load more urgently, especially if there is evidence of leukostasis causing the clinical manifestations previously described. This may be achieved by the temporary use of large doses of hydroxyurea, on the order of 3–6 g daily, and/or by leukapheresis. Leukapheresis may also be considered as a useful temporizing treatment during pregnancy [37, 38].

Prior to the initiation of antileukemic therapy, it is important to discuss and document the implications for future fertility with the patient and possibly his or her partner. It is possible to arrange cryopreservation of spermatocytes and sometimes oocytes, and this is preferably done in liaison with a center specializing in fertility medicine. Although unusual, if blood products are required, CMV-negative products should be administered until the CMV status is available.

Historically, before the development of TKIs, patients were treated with either hydroxyurea or busulfan, both for initial cytoreductions and for long-term control. Although generally well tolerated, therapy was palliative, and neither drug was able to reduce or eliminate the Ph clone and neither changed the inexorable evolution to blast crisis (Fig. 5.1). Hence, all suitable patients were considered for allogeneic transplantation. Trials with interferon-alpha, alone or in combination, were initiated in the early 1980s. The results with these older therapy will be reviewed briefly before detailed discussion of treatment with TKIs, initially studied in the late 1990s and widely available a few years later.

Hydroxyurea (Hydroxycarbamide)/Busulfan (Myleran)

Hydroxyurea, in doses of 2–3 g/day, may be given for a few days or weeks if a TKI is not immediately available [39, 40]. The dose is decreased as the WBC declines. Since its introduction in 1972 and until 2000, hydroxyurea was the "workhorse" therapy for CML. It is highly effective in controlling the hematological abnormalities of this disorder but does not produce any useful cytogenetic responses, even at

high dose [40]. It has the advantage of relatively few side effects but has to be given continuously. Busulfan was also used by many clinicians and had the potential advantage that it could be given intermittently. A randomized comparison suggested an advantage for hydroxyurea [41].

Interferon-Alpha

Since the first reports of its activity in CML in 1983 [42], interferon-alpha has been widely investigated both as a single agent and in combination [43] (Fig. 5.1). There are no convincing data that there were significant differences between Roferon (Roche) and Intron A (Schering Plough). Both products are available in a pen device similar to those used for delivering insulin and a pegylated version which can be given weekly is now available. The precise mechanism of action is unclear although many possible mechanisms have been proposed [44, 45].

A meta-analysis by the CML Trialists' Collaborative Group of seven randomized trials [46, 47] demonstrated a statistically significant survival advantage of IFN (57% at 5 years) over either hydroxyurea or busulfan (42% at 5 years). The initial adverse effects of IFN administered daily include fevers, chills, malaise, arthralgia, and myalgia, sometimes summarized as "flu-like," whereas chronic toxicities include autoimmune-type phenomena, hypothyroidism, Raynaud's phenomenon, connective tissue disorder, and neuropsychiatric disorders. Patients commonly feel very tired and depressed. Elevations of liver enzymes and triglycerides are common.

Interferon in combination with cytarabine has been evaluated in a number of studies. Guilhot et al. reported data from a multicenter randomized trial comparing IFN+ low-dose cytarabine with IFN alone [48]. They reported a significant increase in the rate of major cytogenetic response (41% vs. 24%) and improved survival (85.7% vs. 79.1% at 3 years) in chronic-phase CML patients treated with the combination. The rate of CCyR was low. The main problem was discontinuation of the combined therapy due to side effects. Other investigators have also demonstrated the efficacy of this regimen [49], but other studies have failed to confirm a survival advantage. Interestingly, although most cytogenetic responses were partial, a small fraction of interferon-treated patients achieved persistent complete cytogenetic responses. This combination served as the control arm in the IRIS study which evaluated the benefit of imatinib as initial treatment in chronic phase [50].

Imatinib Mesylate for CML in Chronic Phase

The tyrosine kinase activity of the *BCR-ABL1* oncogene is required for malignant transformation of hematopoietic cells in CML [9, 51, 52]. Imatinib mesylate is a 2′-phenylaminopyrimidine compound designed to inhibit the binding of phosphate donor ATP to the kinase domain (SH1) of the protein. This activity prevents the downstream phosphorylation of the signal transduction proteins involved in leukemogenesis [51, 53–56]. Imatinib inhibits *BCR-ABL1*, *TEL-ABL*, and *ABL* kinase activity and inhibits growth and viability of cells transformed by any of these *ABL* oncogenes. In addition, it was found to inhibit other tyrosine kinases encoded by *PDGF-R* and *c-KIT* as well as cells expressing the p210 BCR-ABL1 protein tyrosine kinase found in Ph + ALL.

Imatinib was developed by Lydon and Druker [57] in collaboration with Ciba-Geigy (now Novartis) in the early 1990s. The compound entered clinical trials in June 1998 and by the end of 1999 it had become clear that imatinib was active in controlling the hematological and clinical features of CML, with high bioavailability as an oral compound. A phase I study demonstrated that at a dosage level of over 300 mg/day by mouth, the drug was able to restore the leukocyte count to normal in patients with IFN-resistant CML in chronic phase with a few patients achieving Ph negativity [36]. The drug was also active in controlling the blast cell count in patients in myeloid blast and lymphoid transformation, but the majority relapsed within 6 months [58].

A series of large phase II studies in interferon-refractory or -intolerant chronic-phase patients confirmed the remarkable efficacy of 400 mg imatinib daily, with high rates of durable CCyR with excellent overall tolerance, resulting in regulatory approval in this patient population [59], as well as in patients with more advanced disease [60–62]. Subsequently, a phase III prospective randomized trial (the so-called IRIS study) was conducted in 1106 previously untreated patients, comparing interferon and cytarabine as per the previous French regimen [48], with imatinib at a dose of 400 mg daily [50]. Fourteen and one-half percent of the patients receiving interferon and low-dose cytarabine achieved a CCyR as opposed to 76% of the patients in the imatinib arm. These differences were highly significant and in terms of side effect profile, tolerability, and quality-of-life measures and rate of major molecular response (MMR) [63], there again were major and significant differences in favor of imatinib [63]. Other trials [64–67], as well as longer term follow-up of the IRIS patients [68, 69], confirmed the durability of these benefits with an estimated event-free survival at 6 years of 83%, and freedom from progression to accelerated or blast phase of 93% on the IRIS trial (Fig. 5.2). Furthermore, responding patients whose disease had not progressed in their first 3 years were extremely unlikely to relapse at a later time and also unlikely to suffer from late-onset side effects. These results were the first to validate the achievement of CCyR as a surrogate for significant and durable long-term benefit of TKI treatment in newly diagnosed patients. Historical comparisons showed a survival advantage from imatinib treatment compared to interferon-based therapies [70, 71].

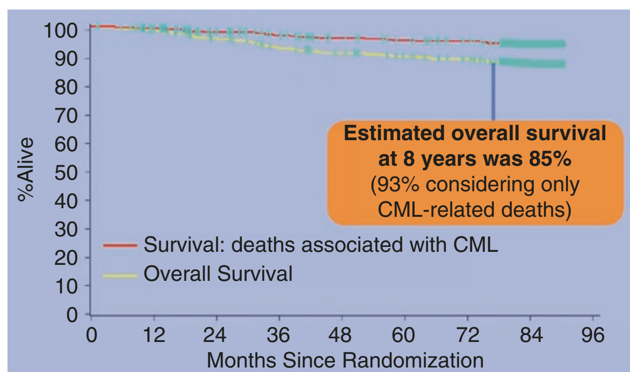


Fig. 5.2 Survival of imatinib-treated patients on the IRIS trial (reprinted with permission from O'Brien SG, Guilhot F, Goldman JM, Hochhaus A, Hughes TP, Radich JP et al. International Randomized Study of Interferon versus STI571 (IRIS) 7-year follow-up: sustained survival, low rate of transformation and increased rate of major molecular response (MMR) in patients (pts) with newly diagnosed chronic myeloid leukemia in chronic phase (CML-CP) treated with imatinib (IM). Blood 2008; 112: 76 (Abstract #186))

Imatinib Dose

Investigators at the MD Anderson Cancer Center in Houston reasoned that clinical results might be improved by starting treatment with a higher dose of imatinib and therefore designed a study in which newly diagnosed patients started treatment with imatinib at 800 mg daily [72]. Many patients are unable to tolerate this higher dosage, but comparison with the results of the standard 400 mg daily showed that CCyRs and major molecular responses (MMR), defined as a 3-log reduction in *BCR-ABL1* transcripts on the International Scale (IS—see below), were achieved more rapidly with the higher dose of imatinib. This experience led other investigators to design studies to compare prospectively 400 mg daily with 600 or 800 mg daily [64, 66, 73, 74]. The large German CML IV trial extended these observations showing that a higher fraction of patients treated with the higher dose achieved a 4.5-log reduction with the higher dose [66]. It does, however, appear that whereas response to the higher dose is clearly more rapid, the longer term results show less convincing superiority for the higher dose and no survival benefit has been demonstrated, likely in part because of the effect of potent second-generation TKIs given after initial imatinib treatment. Thus, at this time, there seems no good reason to alter the "standard" 400 mg initial dosage for adults. Parenthetically, pediatricians have opted to adapt imatinib dosage based on the child's body weight or body surface area [75].

Side Effects of Imatinib

Though patients taking imatinib are spared the more unpleasant side effects associated with conventional cytotoxic drugs, imatinib can still cause a variety of unwanted adverse reactions and some are severe enough to necessitate reducing dosage or discontinuing the drug [76–78]. Among the most prominent non-hematologic side effects are nausea, fluid retention, weight gain, diarrhea, bone pains, rashes, and disturbances of liver function. Most of these symptoms occur early in the treatment course, are of low grade, and can resolve over time. Imatinib can also cause significant cytopenias which almost always improves; occasional patients with anemia may benefit from administration of erythropoietin. An international consortium evaluated more than 800 patients in CyCR for greater than 2 years and then followed for a median of ~6 years. Fatigue and muscle cramps were the most prominent longer term side effects. The findings were very reassuring in that there was no increase in the occurrence of cardiovascular events, organ dysfunction, or other cancers. The overall survival of these patients was identical to that of age-matched normal controls [79]. Another long-term evaluation showed that other medical comorbidities did not influence response or CML-related survival [80]. In children, prolonged use of imatinib may cause skeletal growth velocity and comprehensive long-term follow-up studies are desirable, given the likelihood that most patients will be receiving imatinib indefinitely [81].

Monitoring Responses to Treatment with TKIs

The first evidence of response is reduction of the elevated leukocyte count and resolution of splenomegaly, followed by normalization of a more sensitive measure of residual leukemia, namely the number of Ph-positive metaphases in the bone marrow. The most sensitive test for low levels of leukemia is to measure *BCR-ABL1* transcript numbers in the blood or marrow using a real-time quantitative reverse transcriptase-PCR (RT-PCR or RQ-PCR) [82–84]. Analysis of blood gives results equivalent to those derived from bone marrow, but the specimen needs to be processed within 48 h of collection. Fluorescence in situ hybridization (FISH) used to identify a *BCR-ABL1* fusion gene in interphase cells is more sensitive than metaphase cytogenetics but less sensitive than RT-PCR. Peripheral blood FISH, which correlates very well with marrow cytogenetics, may be used if RQ-PCR for *BCR-ABL1* is not available [85]. In the past, the standard approach was to monitor marrow metaphase cytogenetics until a patient achieves CCyR and then to monitor RQ-PCR for *BCR-ABL1* transcripts at regular intervals to quantify the depth of reduction and to recognize incipient relapse. In patients with a clear-cut marked reduction in RT-PCR levels to a level consistent with at least a CCyR, some clinicians no longer do bone marrows for cytogenetic analysis.

Although RQ-PCR to quantify *BCR-ABL1* transcript numbers is now widely available, it must be remembered that

the technique is demanding and can give both false-positive results (if for example the specimen is accidentally contaminated with material from another patient) and false-negative results (if for example the patient's specimen is degraded during collection and processing). The measurement of a control gene, usually *ABL1*, can assess whether the sample is adequate for analysis and results are expressed as the ratio of BCR-ABL1 to levels of the control gene. Initially, the methods by which results are expressed in different laboratories were not standardized, although most laboratories now express results as a log reduction using the International Scale (IS) which uses a correction factor applied to the "raw" results which are unique to individual laboratories and kits, and which provides standardization and comparability of results among different testing laboratories [86–88].

The IS arbitrarily assigns a baseline value of 100% to each patient [86]. A two-log reduction on the IS (i.e., a value of 1%) roughly corresponds to a CCyR while a greater-than-three-log reduction is termed a "major molecular response" (MMR). Both CCyR and MMR have been used as endpoints in clinical trials (see below). Current PCR technologies permit detection of a single CML cell among ~10^5 normal cells with corresponding IS values of 4.5- to 5-log reductions. In contrast, cytogenetics can detect ~1 Ph + cell among 20 normals and FISH ~1/200 normal cells. If transcripts are not detectable, the term "complete molecular response (CMR)" is sometimes used although this result is still consistent with the survival in a patient's body of perhaps 1×10^7 leukemia cells [88, 89], some of which may be resistant to all currently available TKIs. At very low levels of residual leukemia, the use of a DNA-based PCR technique may be more sensitive than one based on cDNA [89]. Lastly, although highly quantitative, there can be variation in QT-PCR values in duplicate specimen analyzed in the same laboratory, and hence, it is recommended that values be repeated before making decisions about changing therapy.

Resistance to Imatinib

A small proportion of patients who start imatinib in chronic phase, but a larger proportion of those who start the drug in advanced phases, do not respond well initially and require treatment with alternative agents. This is defined as primary resistance. Other patients can respond initially, achieving either a hematological response or a cytogenetic response followed by loss of response. This is defined as secondary resistance. The overall incidence of resistance is obviously time dependent but is of the order of 20–30% in patients who start treatment in chronic phase. The risk of developing secondary resistance appears to diminish with time and patients who have been in CCyR for more than 2 years, and who continue to take imatinib, seem to have an exceedingly low risk of relapse. Treatment failure occurs more commonly in patients with more "advanced" chronic phase as assessed by clinical measures such as the Sokal score, an observation consistent with the lower response rates in patients in accelerated phase. Patients with higher scores who do respond well, however, have long-term outcomes similar to other responding patients.

Resistance can also be classified according to whether it is associated with continuing inhibition of kinase activity of the *BCR-ABL1* oncoprotein or reversal of this inhibition. The mechanisms associated with this reversal of inhibition are not well defined, but some patients have cytogenetic evidence of clonal evolution and occasional examples of amplification of the *BCR-ABL1* gene in patients resistant to imatinib have been identified. The best characterized molecular event in patients with imatinib resistance is the acquisition of point mutations in the kinase domain of the *BCR-ABL1* gene, most commonly evaluated by Sanger sequencing, such that a mutant oncoprotein is produced. The prototype is the T315I mutation whereby a threonine at position 315 in the *BCR-ABL1* protein is replaced by an isoleucine which impedes binding of imatinib in the ATP-binding pocket of the enzyme [90–93]. Other mutations in residues that make contact with imatinib in the P-loop of the enzyme can also be associated with clinical resistance. More than 50 different mutations have been identified in patients who have lost their response to imatinib and other TKIs [94], although such mutations are found in at most 50% of such patients, indicating that other as-yet unidentified molecular events are the direct cause of resistance. Testing for mutations in *BCR-ABL1* should be done whenever a change in therapy is contemplated because of resistance to treatment, since occasionally the mutational profile would suggest using a particular TKI [95, 96]. Mutations are more common in advanced stages of CML and multiple mutations, consistent with progressive genetic instability, can sometimes be identified at the time of progression. In contrast, *BCR-ABL1* mutations are rarely found at diagnosis in chronic phase and mutational testing is not advised at this stage.

Another possible mechanism of resistance is related to increased expression of the P-glycoprotein (Pgp) efflux pump affecting the transmembrane transport of imatinib [97, 98]. Conversely, imatinib is transported into cells by the human OCT1 transporter and low levels of OCT1 have also been linked with poor clinical response to imatinib [99]. In practice, acquired resistance is likely to be multifactorial and mechanisms may differ in different patients. One study identified a gene signature suggestive of disease progression which could be found in patients in clinical chronic phase prior to their disease progression although this approach is currently not in use clinically [100].

In vitro measurement of the concentration of imatinib that will inhibit proliferation by 50% (IC50) of an individual

patient's cells has been studied in an attempt to predict the desirable in vivo imatinib concentration [101]. Two studies suggested that low plasma "trough" levels of imatinib, as defined as the lowest level before the next scheduled dose of imatinib, can correlate with a lower probability of achieving a CCyR [102, 103]. Such low trough levels might be an indication for increasing the prescribed dose of imatinib. There was considerable overlap between the levels in good and less robust responders, and neither approach can be recommended for use in individual patients.

Perhaps most importantly, it must be appreciated that a major cause of an inadequate response or loss of initial response to imatinib may be the simple fact that some patients may not take the prescribed dose on a regular basis, for which there may be a variety of reasons, including forgetfulness, desire to lessen side effects, or desire to save money in countries where the patient has to pay for the drug themselves [104, 105]. Moreover, casual questioning of the patient about his or her adherence to the prescribed dosage may not always yield reliable answers. It is imperative to carefully question patients about compliance before switching treatment or increasing dosage.

Evaluation of Response to Imatinib: European LeukemiaNet Recommendations

In order to optimize the treatment benefit from imatinib, molecular and/or cytogenetic responses should be monitored serially so that changes in therapy can be recommended as needed. The European LeukemiaNet has presented the consensus recommendations of a panel of experts who agreed on a series of criteria for CP patients who started imatinib at 400 mg daily to define treatment "failure," "optimal response," and an intermediate "warning" group who require closer monitoring [106]. The most recent iteration of these recommendations, published in 2013 [107], is summarized in Table 5.5 with an updated version currently being assembled.

It must be emphasized that fluctuations in values are common over time and that there is also considerable inherent variability in the tests themselves. Thus, values near these somewhat arbitrary "cutoffs" should be repeated before changes in therapy are considered. These guidelines may also be used to evaluate the effectiveness of treatment with other TKIs, used either as initial or salvage therapy. Similar guidelines have been offered by the National Comprehensive Cancer Network in the USA (http://www.nccn.org). In earlier years, the treatment change usually involved an increase in imatinib dosage to 600 or 800 mg/day; more recently, switches to newer, more potent TKIs are usually recommended. This approach is supported by a randomized phase II trial which demonstrated superior outcomes with the use of dasatinib in this setting when compared with increased doses of imatinib [108].

Perhaps the most debated aspect of the ELN recommendation concerns the "warning" should patients not achieve BCR-ABL1 ≤ 10% at 3 or 6 months. Numerous studies have shown that a > 1-log reduction (i.e., <10%) is associated with an excellent long-term outcome with few long-term treatment failures, assuming continued compliance with the medication [66, 69, 109–113]. The results are poorer in patients who remain >10% at 3 months, although still >80% of these patients are long-term survivors with continued therapy with either imatinib or other TKIs [66]. Although the temptation is to switch therapy rapidly in patients with slower responses, the only trial which evaluated this approach systematically failed to demonstrate benefit from earlier switching from imatinib to nilotinib [114]. In addition, the 10% number is not an absolute dichotomous decision point in that values of 11% and 9%, for example, are within the range of tests of the same sample done in duplicate. Similarly, the implications of 11% vs. 50% (both >10% on the IS) are certainly different. Thus, tests should be repeated and a pattern or trend established, before therapy is switched to another TKI, particularly at the 3- and 6-month time points.

Some recent studies have suggested that the rate at which the transcripts decrease in the first few months after

Table 5.5 Criteria for definition of response based on European LeukemiaNet recommendations [107]

	Failure	Warning	Optimal response
3 months	Non-CHR and/or Ph+ >95%	BCR-ABL1 > 10% and/or Ph + 36–95%	BCR-ABL1 ≤ 10% and/or Ph + ≤35%
6 months	BCR-ABL1 > 10% and/or Ph+ >35%	BCR-ABL1 1–10% and/or Ph + 1–35%	BCR-ABL1 < 1% and/or Ph + 0
12 Months	BCR-ABL1 > 1% and/or Ph+ >0	BCR-ABL1 > 0.1–1%	BCR-ABL1 ≤ 0.1%
18 months	Less than CCyR		MMR (3-log reduction in transcripts)
Any time	Loss of CHR	CCA/Ph– (−7, or 7q–)	BCR-ABL1 ≤ 0.1%
	Loss of CCyR		
	Kinase domain mutation insensitive to Imatinib		
	Confirmed loss of MMR		
	Clonal evolution		

CHR complete hematological response, *CyR* cytogenetic response, *CCyR* complete cytogenetic response, *MMR* major molecular response

TKI therapy is begun, sometimes termed the "velocity of elimination," might be a more sensitive means of distinguishing between good and poor responders. This approach requires better standardization of the transcript levels at the time of diagnosis and is not broadly applicable at this time [111, 115].

Managing the Patient in Whom Imatinib Has Not Been Sufficiently Effective

Changes in therapy after treatment with imatinib can be prompted by the development of intolerable side effects, failure to obtain adequate reduction in BCR-ABL1 transcripts as defined above, or less commonly obvious disease progression. It is critical to assess patient compliance with the imatinib before attributing treatment failure to drug resistance [104]. If a switch in therapy is contemplated, assessment of BCR-ABL1 mutations should be done because occasionally the type of mutation will suggest the use of a specific TKI with better activity against that particular conformational change in the BCR-ABL1 [116–118]. Most often however, either no mutation is detected or the sensitivities to other TKIs are predicted to be similar, and the choice of second TKI is predicated on issues such as convenience of therapy and side effect spectrum.

So-called second-generation TKIs were synthesized and entered the clinic remarkably rapidly after the discovery of imatinib. All of these agents are orally bioavailable, were initially evaluated and approved by regulatory agencies for use in patients with treatment failure or intolerance to imatinib, and were subsequently tested in comparative trials with imatinib as initial therapy. All are administered orally. These agents have never been directly compared with each other in randomized trials, but the overall response rates of dasatinib, bosutinib, and nilotinib are similar in imatinib-relapsed/refractory/intolerant patients. CCyR rates of ~40–50% which, if achieved, are generally sustained for many years, can be anticipated, with the highest response rates, as might be expected, in patients switched for intolerance [119–125]. Response rates are lower with "third-line" TKI treatment although sustained responses can also be achieved with a switch to an alternative TKI. Ponatinib is the most potent of these drugs when used in the more advanced setting but has a very high rate of cardiovascular side effects as described below [126, 127]. Because of the lower response rates with second-line treatment, many clinicians evaluate for possible allogeneic transplant, reserving transplant for patients poorly responsive to the subsequent treatment.

The side effect profile varies considerably among the different TKIs, presumably because all inhibit multiple tyrosine kinase pathways in addition to BCR-ABL1 signaling. All can be associated with usually transient rash, diarrhea, fatigue, headache, elevations of liver transaminases, and pancreatic enzymes, with occasional overt pancreatitis, but each also can produce side effects relatively unique to that agent.

Dasatinib

Dasatinib (Sprycel) is an inhibitor with activity against a range of tyrosine kinases including SRC and SRC family kinases. In vitro studies showed that it was 325 times as potent as imatinib against BCR-ABL1 [128]. It is effective in producing major cytogenetic responses in ~40% of the patients still in chronic phase who develop resistance to imatinib [108, 129]. A randomized study evaluating different doses and schedules of dasatinib identified 100 mg/day as the preferred starting dose, although many patients wind up taking lower doses long term with continued effectiveness [122]. These responses can be sustained, and with follow-up of 7 years, the PFS and OS were ~42% and 65%, respectively, using the 100 mg dose [130].

Dasatinib causes more profound cytopenias than does imatinib, and can be associated with QTc prolongation, and pleural effusions can develop in more than 20% of patients [108, 130]. Effusions are more common in patients with more advanced CML and those with prior cardiac disease. Most are asymptomatic and can resolve spontaneously or with dose reduction, but some require thoracentesis and occasionally a switch to another TKI. Effusions are usually detected within the first few months of dasatinib treatment but can occur after many months or years of treatment and patients should be advised to contact their CML care giver should symptoms of shortness of breath or chest discomfort develop. Pericardial effusions have also been noted and a small number of patients have been described with pulmonary hypertension, clinically indistinguishable from "primary" pulmonary hypertension. These can resolve with dasatinib discontinuation but fatalities have been reported [131, 132]. The mechanism(s) responsible for these fluid retention and vascular changes are not known. Lastly, ~30% of CML patients develop a proliferation of T/NK cells within the first few months of treatment which in some patients can persist for years [133–136]. There is a suggestion that the rates of CyCR and PFS may be higher in such patients, suggesting an immunomodulatory effect [137], but these findings need further confirmation.

Nilotinib

Nilotinib (Tasigna) is a chemically modified form of imatinib with greater potency and selectivity for *BCR-ABL1* than imatinib [138]. A large phase 2 study in 321 patients

resistant to or intolerant of imatinib demonstrated a 45% CyCR rate with PFS of 57% and OS of 78% after 4 years of follow-up using a dose of 400 mg twice daily [139, 140]. Nilotinib should be taken on an empty stomach, at least 2 h apart from meals. More recent trials have suggested that the recommended dose should be 300 mg twice daily [141].

Although nilotinib is well tolerated overall, patients have to be monitored carefully during the first 1–2 months for hepatic transaminase elevations and for clinical or subclinical pancreatitis manifested by elevations of lipase and amylase. These abnormalities are usually transient and subclinical, and most patients can be restarted on nilotinib after the values normalize. There is a "black box" warning on the nilotinib label because of a few cases of sudden death occurring early in the nilotinib trials. Fortunately, this was a very rare event in the thousands of patients treated subsequently, although monitoring of the QTc interval is recommended after nilotinib is started. Lastly, the more recent randomized ENESTnd trial in newly diagnosed patients with CML trials demonstrated a somewhat alarming incidence of >15% of cardiovascular events including myocardial and cerebrovascular ischemia and infarction as well as peripheral arterial occlusive disease [141, 142]. The incidence of these problems in imatinib-treated patients was ~1%. This experience confirmed earlier case reports describing peripheral arterial occlusive disease in nilotinib recipients [143]. The mechanism is not known although nilotinib can precipitate or exacerbate diabetes. These issues need to be considered in individual patients when deciding among different TKIs and patients receiving nilotinib should have other vascular risk factors such as hypertension, hypercholesteremia, smoking cessation, and diabetes monitored and treated as needed. Many clinicians also prescribe prophylaxis with aspirin, although there are no prospective data supporting this approach [144].

Bosutinib

Bosutinib (SKI-606, Bosulif), a 7-alkoxy-3-quinolinecarbonitrile, functions as a dual inhibitor of SRC and ABL kinases, and preclinical studies demonstrated a high antiproliferative activity in human and murine CML cell lines [145, 146]. Bosutinib was recently approved in the USA for the treatment of imatinib-resistant/intolerant patients as well as some patients refractory to either nilotinib or dasatinib, based on a phase 2 trial using a dose of 500 mg/day, which demonstrated a major cytogenetic response rate of 40% and OS of 78% with approximately 4 years of follow-up [119, 123, 124, 147]. Newer studies with bosutinib utilize a dose of 400 mg/day. Diarrhea and transaminase elevations occurring during the first few months of treatment are the most common toxicities, sometimes requiring dose reductions and/or temporary cessation of therapy [148].

Ponatinib

Ponatinib is a potent *BCR-ABL1* developed as a scaffold (unlike the other TKIs) designed to make a hydrogen bond with the T315 mutant oncoprotein [91, 127, 149, 150]. Ponatinib adapts to such steric hindrance by virtue of a long and flexible ethynyl tricarbon linker. X-ray crystallographic analysis of the ponatinib-murine T315I ABL complex revealed that the compound binds to the complex in the inactive conformation, similar to imatinib, and interacts via hydrogen bonds at five distinct amino acid residues. In vitro studies demonstrated potent activity against cell lines resistant to other TKIs and large phase 2 studies were conducted in patients resistant to therapy with nilotinib or dasatinib. The phase 2 PACE trial reported major cytogenetic responses in 51% of the 203 patients with chronic-phase CML resistant or intolerant to dasatinib or nilotinib and in 70% of the 64 patients in chronic phase with T315I mutations. Approximately 90% of responses were sustained at 1 year and the drug was effective across a broad range of *BCR-ABL1* mutations [126].

Ponatinib was approved for use in this patient population as well as for patients in more advanced-stage CML. Short-term toxicities included skin rash, hypertension, and occasional pancreatitis. However, longer term follow-up demonstrated a disturbing incidence of predominantly arterial cardiovascular events including strokes, myocardial infarction, peripheral arterial occlusive disease, and heart failure [144]. At least 27% of study participants experienced such side effects and although cardiovascular events were more common in older patients with predisposing risk factors, some younger patients without apparent vascular issues were also affected [151]. The original studies used a dose of 45 mg/day and more recent studies are in progress evaluating lower doses and/or rapid reduction of dose as soon as responses are detected. The mechanism of the vascular toxicity is not known [144]. The drug was initially withdrawn from the market but is now available for use in highly selected patients and in particular those with the T315I mutation for which there is no other effective therapy [152, 153]. If patients are responding, the lowest effective dose should be used and the author follows some patients in sustained molecular response using 15 mg/day.

Omacetaxine (Homoharringtonine)

Omacetaxine mepesuccinate (Homoharringtonine HHT) is a novel plant cephalotaxine alkaloid originally derived from the *Cephalotaxus fortunei* tree [154–156]. Omacetaxine is approved in the USA for the treatment of chronic-phase CMl resistant to two TKIs, including patients with the T315I mutation. The recommended dose and schedule are 1.25 mg/m^2

subcutaneous injection twice daily for 14 days of a 28-day cycle for the induction phase and 1.25 mg/m^2 subcutaneous injection twice daily for 7 days of a 28-day cycle for maintenance. Rates of complete hematologic, major cytogenetic, and complete cytogenetic response were 77%, 23%, and 16%, respectively [109, 157]. The main side effects include significant cytopenias, infection, diarrheas, fatigue, and fever. Median PFS was ~7 months. Omacetaxine is sometimes used as a bridge to transplantation in patients in more advanced stages, although hydrea and myleran are also used for disease control and palliation in this situation.

Initial Therapy of Chronic Phase with Newer TKIs

Shortly after approval of the second-generation TKIs for imatinib refractory patients, randomized trials were initiated using these drugs as initial treatment in chronic phase. In the Dasision study, previously untreated patients were randomized to receive either 100 mg daily of dasatinib or imatinib, 400 mg daily [110, 158]. In the ENESTnd study, two different doses of nilotinib, 300 mg twice daily and 400 mg twice daily, were compared with imatinib 400 mg daily [141, 142, 159]. The primary endpoints were CCyR and MMR after 12 months of treatment. In both studies, cytogenetic and molecular responses were achieved more rapidly than with imatinib and in both studies the early rate of progression from CP to advanced phase was higher in the imatinib arm than in any of the three test arms. Based on these results, both dasatinib and nilotinib were approved by regulatory agencies as initial treatment in chronic phase.

A trial of similar design (BELA) evaluating bosutinib was also completed, and although overall results were actually very similar to the other trials, bosutinib has not yet been approved as initial therapy [160, 161]. A repeat trial using a dose of 400 mg of bosutinib was recently completed and demonstrated higher rates of CyCR in the bosutinib treated patients.

Lastly, a trial comparing ponatinib and imatinib was stopped before completion when the cardiovascular side effects of ponatinib became apparent [162]. Even in this trial, with only short follow-up, 6% of ponatinib-treated patients experienced severe cardiovascular events (Table 5.6).

These studies have appropriately influenced discussions of the preferable first-line treatment of chronic phase. Cross-study comparisons cannot be done because of some differences in statistical methods, definitions of endpoints, duration of follow-up, and most importantly variability in the details of follow-up and types of treatment after patients went "off study" or switched from their original randomized treatment. All of these trials were sponsored by pharmaceutical companies. A study comparing imatinib and dasatinib done in the USA, and coordinated by the Southwest Oncology Group, was similar in design to Dasision and reported very similar results [113].

The overall results of these studies are very similar and a number of general statements can be made:

- OS was very high with no differences according to initial treatment, although most studies reported slightly higher rates of "CML-related" deaths in the imatinib-treated patients.
- With longer follow-up, 40–50% of patients were no longer receiving their initial treatment. Switches were made because of side effects and occasionally for disease progression (somewhat more commonly in the imatinib groups) and presumably most of these patients received alternative TKIs. Details of secondary treatment are generally not available, but it is important to emphasize that one must consider the totality of initial and subsequent therapy in evaluating overall results. Given the excellent OS and low rates of progression, it is clear that subsequent treatment has a critical role in achieving these outcomes.
- Transformation to AP/BP occurred more frequently with imatinib treatment. Almost all these events occurred within the first 1–2 years and were quite uncommon with longer follow-up, although the overall rate of transformation was low with all treatments. Information about whether transformations developed primarily in patients with "advanced" chronic phase with high-risk features is not available. This is important because many experi-

Table 5.6 Summary of long-term results

	Dasision (*n* = 519) (Minimum of 5 years of follow-up)		ENESTnd (*n* = 846) (Minimum of 5 years of follow-up)			BELA (*n* = 502) (Minimum of 2 years of follow-up)	
	IMAT	DAS	IMAT	NIL 300	NIL400	IMAT	BOS
CyCR (%)	80%	85% @ 18 months	77%	87% @ 24 months		79%	80% @ 24 months
MMR (%)	64%	76	60%	77	77	49%	59% @ 24 months
AP/BC (%)	7.3	4.6	4.8	1.3	0.7	5.5	1.6
PFS (%)	85	86	91	95.8	92.2	88	92
OS (%)	90	91	91.7	93.7	96.2	95	97
Receiving initial therapy (%)	63	61	50%	60	62	71	63

enced clinicians now utilize second-generation TKIs in preference to imatinib in such patients, based on the results of the randomized trials.

- All studies showed that results were inferior in patients with transcripts >10% at 3 months, independent of treatment arm, although a higher fraction of patients achieved this "milestone" with the second-generation TKIs. There is no information about subsequent approaches to treatment in such patients and the problems of relying on a single IS value to change treatment have been described above.
- The "depth" of molecular response continued to improve over time and all studies noted lower rates of "deep" reductions >4 and 4.5 logs in imatinib-treated patients. There is speculation that this may permit more patients treated with second-generation TKIs to successfully discontinue treatment but there are as yet no data addressing this issue. It should also be noted that all the response rates reported in the table utilized the cumulative incidence statistic and information as to whether these deep responses persisted on repeated testing is not available.

The decision about which TKI is preferable as initial therapy must also consider the unique toxicity profiles of the drugs. Some notable issues include the following:

- There was an appreciable incidence of **cardiovascular events** of approximately 15–20% in nilotinib-treated patients which continues to increase over time, compared to 3–4% in imatinib-treated patients. Rates in the Dasision trial were ~5% vs. 2% in dasatinib- and imatinib-treated patients, respectively. Glucose intolerance and hypercholesteremia were also more common with nilotinib compared to imatinib. Recommendations about concurrent treatment of cardiovascular risk factors and patient selection for treatment with nilotinib have recently been published [163–165], but it is not known if these approaches will be effective in reducing the incidence of cardiovascular side effects.
- **Pleural effusions** developed in ~28% of dasatinib-treated patients compared to 0.1% in the imatinib group. Most such patients could continue dasatinib with temporary discontinuations and dose adjustments. **Pulmonary hypertension** with clinical manifestations was detected in 5% of dasatinib-treated patients.
- Eleven **infectious deaths** were reported in the dasatinib group in the Dasision trial, compared to one in the imatinib group. There is no apparent explanation for this discrepancy.

The debate about the best initial treatment continues [166]. It is apparent from these studies that the second-generation TKIs are more potent than imatinib in that the rate of initial response is faster, and the depth of response may be greater with a likely lower (albeit still very low) rate of initial disease progression, but with equivalent overall survival. Longer term toxicity data with bosutinib are lacking, but it is clear that both nilotinib and dasatinib have important issues unique to each agent, whereas the long-term follow-up data with imatinib are very reassuring [79, 80, 106]. Certainly, preexisting medical problems in individual patients have to be an important consideration. In any event, it is likely that with the increasing availability of generic imatinib, insurers and other payors will be directing, if not mandating, initial treatment with imatinib. Fortunately, the results of these randomized trials provide reassurance that this is a most reasonable approach for the overwhelming majority of newly diagnosed patients. In addition, a large trial of ~1500 patients conducted by the German CML group which evaluated different approaches using imatinib confirmed these excellent long-term outcomes [66].

Stopping Imatinib in Responding Patients: The Concept of "Treatment-Free Remission"

A small number of patients treated with interferon-alpha in the 1990s achieved durable CCyR which has continued for many years after the interferon was stopped [47]. In 2007, Rousselot and colleagues reported details of 12 CML patients in France who received imatinib as primary treatment or after prior treatment with interferon and achieved CMR [167]. These patients had stopped their imatinib for different reasons after 2 or more years in CMR; six had relapsed at the molecular level and six were still in CMR at the time of the report. These observations were extended in the STIM ("Stop Imatinib") study of 100 patients who had undetectable transcripts over a number of years using highly sensitive PCR assays. Approximately 50% of patients had a molecular relapse, almost all within 6 months of treatment cessation [168]. All relapsing patients promptly responded to reintroduction of imatinib which was restarted when MMR was lost (>0.1 on the IS) [169, 170]. Remarkably, only rare recurrences were detected in the remainder of the patients, with the latest update at a median follow-up of 4 years. The relapse rate was lower in patients who were PCR undetectable for >8 years. Other studies have shown similar results [171]. Importantly, discontinuation should only be considered in patients with consistently very low to undetectable transcript levels for at least a few years and many trials, some of which use different durations and depth of "negativity" prior to cessation, are in progress. All trials mandate careful molecular monitoring for the first 12 months with continued intermittent long-term monitoring thereafter, because it is not known if very late relapses will occur. It has been postulated that the long remissions may be due to immunologic control of any residual CML stem cells, but the precise mechanisms are unknown.

Whether such patients can really be regarded as "cured" is debatable but undoubtedly one major target today should be to find strategies that will enable us to increase the proportion of patients who may safely stop treatment [172, 173].

An interesting "withdrawal syndrome" characterized by musculoskeletal and articular discomfort and pain has been described in an appreciable fraction of patients who discontinued TKIs. The symptoms are usually mild and transient and easily managed with nonsteroidal anti-inflammatory agents, although persistence of clinically significant achiness can occur in a few patients, which may or may not improve with restarting TKIs [174, 175].

Imatinib and Other TKIs During Pregnancy

Preclinical studies based on animal data suggested that imatinib and other TKIs could be teratogenic in certain circumstances and women taking imatinib have been routinely advised to take steps to avoid conception. Nonetheless, some women have conceived while taking imatinib and in most cases where the pregnancy went to term the baby appeared to have been normal. However, certain specific developmental abnormalities including hypospadias, exomphalos, and defective skeletal formation have been seen more often than would have been expected in women not taking imatinib [176, 177] and the advice to avoid pregnancy while being treated with the drug must be upheld.

Therefore, counseling a woman already on imatinib who wants to start or to enlarge her family is difficult, and generally clinicians continue to advise against pregnancy. If the patient has achieved a deep response, stopping imatinib to allow the patient to conceive and carry the child without exposure to imatinib is a consideration, but this approach almost certainly puts the patient at increased risk of relapse and possibly disease progression. Interferon-alpha is not known to be teratogenic and has been considered to be an alternative, particularly for women who become pregnant while receiving a TKI. There are few published data evaluating this approach, however. Women who become pregnant while receiving imatinib should contact their physicians immediately. Available evidence suggests that patients who stop imatinib and then relapse respond as well to reintroduction of imatinib as they did originally [170]. Less is known about the possible harmful effect of imatinib on male spermatogenesis and small case series have shown a very low rate of fetal abnormalities in children whose fathers were taking imatinib [178].

Advanced-Phase Disease

In the past, patients who presented in accelerated and blastic phases were treated with a higher starting dose of imatinib of 600 mg daily [61], although the use of dasatinib or nilotinib is now preferred in such patients. In general, advanced-phase patients respond much less well to imatinib than those who start treatment in chronic phase with low rates of CyCR and high rates of relapse should response be achieved. However, patients who satisfy the criteria for "acceleration" are in fact heterogeneous: at one end of the spectrum the term covers patients whose leukemia is only slightly more advanced than late chronic phase while in others the leukemia may be verging on blastic phase. Patients with "early" accelerated phase may obtain long-term responses to imatinib as a single agent, while others usually have much shorter responses [61]. Patients presenting in blastic transformation need a much more aggressive initial strategy. All patients in AP/BP should be evaluated for allogeneic stem cell transplantation while they are receiving their initial TKI treatment.

It can sometimes be difficult to distinguish between patients in lymphoid blast phase at the time of diagnosis, from de novo Ph + acute lymphoblastic leukemia (ALL). Combining a TKI with standard anti-ALL treatment is the best initial approach [179]. Once remission is achieved, maintenance treatment with cytotoxic drugs together with a TKI can then be continued. Neuroprophylaxis is also advisable. For patients presenting in myeloid blast phase, the combined use of a TKI with therapy appropriate to AML may be the best approach. AML-like chemotherapy alone produces few complete remissions [180]. In both circumstances, allogeneic stem cell transplantation should be done in all suitable patients, preferably while the patient is in complete remission. Most centers continue the use of a TKI after engraftment, but no comprehensive studies about the duration of such maintenance TKI are available.

Allogeneic Stem Cell Transplantation

Until the advent of TKIs, allogeneic stem cell transplantation was the recommended initial approach for patients who were relatively young and had suitable donors, with rates of disease-free survival approximating 70%, with very few late relapses [181]. Currently, allogeneic SCT is reserved for those with advanced-stage disease or those in whom treatment with second-generation TKIs was not successful. More details about conditioning regimens and donor selection are presented in other chapters. *BCR-ABL1* transcripts should be monitored serially for the first few years after transplant. It is important to recognize that there can be transient *BCR-ABL1* positivity in the first year posttransplant in patients who are ultimately destined *not* to proceed to overt relapse [181]. Two or more consecutive samples with increasing numbers of *BCR-ABL1* transcripts are required at a minimum to establish a relapse. For patients with confirmed relapse after allogeneic SCT, options for treatment include the withdrawal of immunosuppression, use of a TKI to which the patient has not previously been exposed, and/or donor lymphocyte infusions.

Summary

The therapy of CML has been revolutionized over the last 15 years with the advent of TKI therapy, such that the survival of chronic-phase patients responding to TKI treatment approximates that of age-matched controls. There is now the prospect that some patients might be able to stop TKI therapy and remain in remission and possibly even be cured in the longer term. Over the next decade, research will focus on evaluating how to use the various TKIs in the most cost-effective way to further improve the long-term outcome and quality of life of patients with CML. Stem cell transplantation has a minor, but still important, role in the small number of patients for whom TKIs are not effective or tolerated.

Acknowledgments This section is an extensive update of the chapter from the earlier edition authored by Drs. Stephen O'Brien and John Goldman and is dedicated to the memory of John Goldman who was a pioneer and leader in our understanding of the biology and therapy of CML and a valued friend and colleague.

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Etiology and Epidemiology of Chronic Lymphocytic Leukemia

6

Helen E. Speedy, Daniel Catovsky, and Richard S. Houlston

Introduction

Chronic lymphocytic leukemia (CLL) is an indolent malignancy resulting from an accumulation of CD5-positive neoplastic B-cells, characterized by a low rate of proliferation. CLL accounts for approximately 30% of all newly diagnosed leukemia cases each year and is the most common form of adult leukemia in Western countries [1]. Although still an incurable malignancy, recent studies have identified over 30 common genetic variants that are associated with the risk of developing CLL. Deciphering the mechanisms by which these variants influence disease risk represents the next challenge in studies of CLL susceptibility and will be key in advancing our understanding of its biological basis.

Descriptive Epidemiology

CLL is primarily a disease of later life with a median age of diagnosis in European populations of around 70 [1]. Two key features of the disease have hampered the acquisition of descriptive data on CLL. Firstly, CLL is often encountered as a chance (and often late) diagnosis and this in turn can be merely a consequence of healthcare provision rather than any true difference in disease incidence between countries. Secondly, many epidemiological studies have failed to distinguish B-cell disease including prolymphocytic leukemia and possibly lymphocytic lymphomas as part of CLL. Even accepting these caveats it is apparent that the CLL incidence rates are nearly twice as high in men as in women and that rates vary considerably throughout the world.

CLL occurrence is highest in Europe and European populations elsewhere in the world, with low rates in South and East Asia and sub-Saharan Africa (Table 6.1). The lowest recorded rates of CLL come from Japan. The observed

Table 6.1 World incidence rates of chronic lymphocytic leukemia

Race/ethnicity	Male	Female
All races	6.3	3.3
White	6.7	3.5
Black	4.9	2.4
Hispanic	2.7	1.6
Asian/Pacific Islander	1.7	0.7
American-Indian/Alaska Native	1.7	1.3
Country	**Male**	**Female**
Canada	3.7	1.8
Denmark	3.4	1.5
New Zealand	3.0	1.5
New South Wales, Australia	2.8	1.4
Tarragona, Spain	2.4	0.9
England and Wales, UK	2.3	1.1
The Netherlands	2.2	1.0
Turin, Italy	2.2	0.9
Israel Jews	2.1	1.1
Harare, Zimbabwe, Africa	1.7	1.3
Mumbai, India	0.6	0.3
Cali, Colombia	0.5	0.2
Shanghai, China	0.2	0.1
Osaka, Japan	0.1	0.0

The incidence rates are age adjusted and show cases per 100,000 per year. Race/ethnicity rates are based on Surveillance Epidemiology and End Results data on cases diagnosed in 2009–2013. Data for the incidence rates by country used with permission from Parkin DM, Whelan SL, Ferlay J, Ragmand L, Young J (eds): Cancer Incidence in Five Continents, Volume VII. IARC Sci. Publ., 1997

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30-fold variation in national rates has led many researchers to investigate a genetic basis for CLL risk. This notion is reinforced by the observation that the incidence of CLL remains low in Asians, even in those born in the United States, suggesting that genetic susceptibility rather than environmental or lifestyle factors are the greater determinant of risk [2, 3].

Environmental-Lifestyle Risk Factors

Although several environmental risk factors for acute myeloid leukemia are well recognized, such as exposure to benzene [4], smoking [5], and ionizing radiation [6], information regarding the role of chemical exposures in the development of CLL is very limited and no robust associations have so far been documented. In fact, there does not seem to be a clear association between CLL risk and any of the exposures that commonly cause other types of cancer.

Links with agricultural occupations or agricultural chemicals probably provide the strongest leads to date for environmental risk factors [7, 8]. These associations have been observed in several studies of different designs and in different geographical locations, but very few have evaluated specific agricultural agents. Excesses of CLL noted in studies of the rubber [9] and petroleum [10, 11] industries have raised the possibility of links with benzene and other solvents, but such associations remain essentially unvalidated. Other occupations that have been considered as potential risk sources for CLL include mining [12], or those with exposure to asbestos [13] and certain chemicals [14], but again no conclusive etiologic links exist.

Ionizing radiation has been implicated as a cause of most forms of leukemia for several decades, but a number of studies of highly exposed populations have not indicated an association with CLL. The justification for concluding that the risk of CLL is increased by exposure to ionizing radiation has been challenged owing to considerations such as the low background incidence rate of CLL in some studies on which the presumption is based, and the anticipated long latency between initiation and death from CLL [15]. However, a follow-up study specifically taking into account these and other confounding factors did not find a consistent association between radiation and CLL [16].

Immune Dysfunction as a Risk Factor

Intuitively, links between genetics and immune dysfunction as a possible basis for CLL are highly attractive. One study reported that infection is a constant risk in CLL that is associated with shortened survival [17]. It seems apparent that CLL tumor cells utilize immunosuppressive mechanisms to evade immune recognition. Although CLL cells express tumor antigens that can be presented by major histocompatibility complex class I and class II molecules, an effective immune response is not elicited against the tumor cells [18, 19]. However, there is no compelling evidence linking infection by human T-cell lymphotropic virus, human immunodeficiency virus, or immunosuppression following organ transplantation with CLL [20].

A variety of prior medical conditions have been reported to confer an increased risk of CLL including scarlet fever, bronchitis, and rheumatoid arthritis [21]. However, no consistent association has yet emerged and these assertions must be considered as unreliable.

Familial Clustering of CLL

Over the last seven decades more than 100 families have been reported in the literature in which clustering of CLL has been documented. While not exclusively a consequence of genetic predisposition, familial aggregation provides strong evidence for inherited genetic factors playing a role in disease development. In a number of the families reported, CLL co-segregates with other B-cell lymphoproliferative disorders (LPD), such as Hodgkin's lymphoma (HL), suggesting that part of the familial predisposition could be mediated through pleiotropic mechanisms [22–24].

Eight epidemiological case-control and cohort studies have systematically enumerated the risk of relatives of CLL patients developing CLL or other LPDs [21, 22, 25–29]. The largest and most comprehensive of these was based on an analysis of 9717 CLL cases and 38,159 controls ascertained through the Swedish Cancer Registry [27]. This study firmly underscored the notion that CLL is characterized by a high familial relative risk (RR); the RR of CLL in first-degree relatives of cases was increased 8.5-fold. Furthermore, the risk of other non-Hodgkin lymphoma (NHL) in these relatives was increased 1.9-fold. Evaluating NHL subtypes revealed a striking excess of indolent B-cell NHL, specifically lymphoplasmacytic lymphoma/Waldenström macroglobulinemia and hairy cell leukemia [27]. These findings substantiate a relationship between the risk of CLL and other LPDs which has anecdotally previously been noted in case reports of single families and that may reflect the pleiotropic effects of an inherited predisposition.

In familial CLL, the proportion of affected females is higher when compared to sporadic CLL. Females might therefore have more predisposition genes or their genes might be more penetrant than those of males. The relatives of affected females probably share the same predisposition genes, which increase their genetic liability, accounting for the higher proportion of familial cases among females compared to males.

The phenotype of earlier age of onset and increased risk of second tumors is a classical feature of many familial cancers. An early survey of 28 CLL families suggested that familial cases present around 10 years earlier than sporadic cases, implying a more aggressive clonal expansion [30]; however, more recent studies provide little support for such an assertion [31]. Anticipation, the phenomenon of intensified clinical severity and earlier age of onset with each successive generation, has been reported for CLL, with mean declines between parents and offspring being as many as 22 years [32–34]. However, findings were based on data from families ascertained for genetic studies, which are enriched for younger cases, thereby introducing bias through censoring or cohort effects. In a study using Swedish registry data where corrections were made for possible sources of bias there was little evidence to support anticipation in CLL [35].

Models of Inherited Genetic Susceptibility

The observation of large families segregating CLL in an apparent Mendelian fashion provided a strong rationale for searching for moderate–high-risk gene mutations. To date, five linkage scans of CLL families have been performed [36–40]; however none has provided robust evidence for the existence of a single major locus conferring susceptibility to the disease. The premise of these studies is that in a proportion of families with CLL, chromosomal regions that harbor mutations conferring a substantive disease risk will segregate with the affected family members.

The failure of linkage analysis to identify a high-impact CLL risk locus led to a reappraisal of the inheritance pattern in CLL and an increased interest in a polygenic model of disease susceptibility (Fig. 6.1).

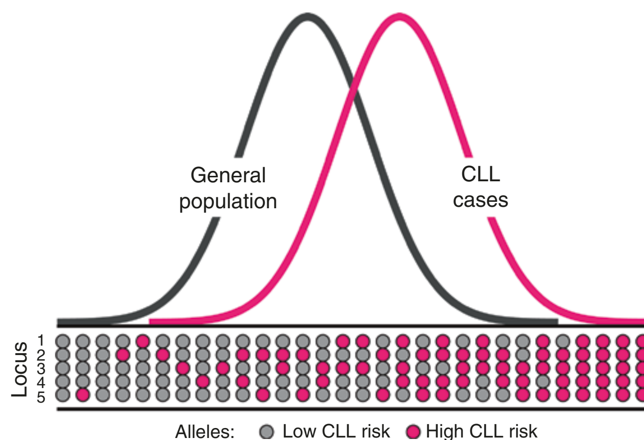

Fig. 6.1 The polygenic model of disease susceptibility. The distribution of alleles at CLL risk loci in both cases and controls follows a normal distribution. However in cases there is a shift toward an increased number of high-risk alleles. For clarity, only five risk loci are illustrated

This model, which is based on disease risk being a function of the inheritance of multiple low-risk variants, is amenable to interrogation by a genome-wide association study (GWAS) design. This approach has proved particularly successful in determining genetic risk factors for CLL and is discussed in detail below.

Common Genetic Susceptibility to CLL

The search for low-risk alleles for CLL has centered on association studies, where the frequencies of common variants (usually single-nucleotide polymorphisms, SNPs) are compared between cases and controls. Initial efforts were small-scale studies that employed a candidate gene-based approach. No susceptibility alleles have been unequivocally identified by this method which is hampered by the inherent statistical uncertainty that results from studying just a few hundred cases and controls and has only limited power to reliably identify genetic determinants conferring modest but potentially important risks [41]. Furthermore, without a clear understanding of the biology of predisposition the definition of suitable genes for the disease is inherently problematic making an unbiased approach to loci selection highly desirable.

The publication of International HapMap Consortium catalog of common human genetic variation [42] and the subsequent widespread manufacture of high-density SNP genotyping arrays revolutionized the case-control association study design. The GWAS approach assesses disease association in an unbiased manner, by comparing the frequencies of thousands of SNPs, surveyed across the genome, in a large number of cases and controls. In the past 8 years, GWAS have identified 35 independent variants at 31 genetic loci that are significantly associated with CLL risk (Table 6.2, [43–51]).

The effect size of the individual variants identified by GWAS is modest, with relative risks in the order of 1.16–1.87 per allele (Table 6.2). However, it is worth noting that the common occurrence of the risk alleles (>5% frequency) means that their cumulative burden in the population and contribution to disease incidence is high. Moreover, the burden increases with increasing numbers of variant alleles and for the 2% of the population who carry 13 or more risk alleles; the risk of CLL is increased approximately eightfold. Grouping cases and controls according to the number of risk alleles carried illustrates that the effect of these loci fits the polygenic model of inheritance (Fig. 6.1), with the number of risk alleles following a normal distribution in both cases and controls, but with a shift toward a higher number of risk alleles in cases [45].

Latest estimates suggest that common variation can explain up to 57% of the familial risk of CLL. To date, the

Table 6.2 SNPs associated with CLL risk in published genome-wide association studies

Locus	Published SNP	Nearest gene(s)	Study	SNP type	Reported OR[a]
2p22.2	rs3770745	*QPCT*	[44]	Intronic	1.24
2q13	rs13401811	*ACOXL, BCL2L11*	[44]	Intronic (*ACOXL*)	1.41
	rs17483466	*ACOXL, BCL2L11*	[47]	Intronic (*ACOXL*)	1.39
	rs9308731	*ACOXL, BCL2L11*	[43]	Intronic (*BCL2L11*)	1.19
2q33.1	rs3769825	*CASP8*	[44]	Intronic	1.19
2q37.1	rs13397985	*SP140*	[47]	Intronic	1.41
2q37.3	rs757978	*FARP2*	[45]	Missense	1.39
3p24.1	rs9880772	*EOMES*	[43]	Intergenic	1.19
3q26.2	rs10936599	*MYNN*	[51]	Synonymous	1.26
3q28	rs9815073	*LPP*	[43]	Intronic	1.18
4q25	rs898518	*LEF1*	[44]	Intronic	1.20
4q26	rs6858698	*CAMK2D*	[51]	Intergenic	1.31
5p15.33	rs10069690	*TERT*	[51]	Intronic	1.20
6p25.3	rs872071	*IRF4*	[47]	3′-UTR	1.54
6p25.2	rs73718779	*SERPINB6*	[43]	Intronic	1.26
6p21.32	rs674313	*HLA-DRB1*	[49]	Intergenic	1.87
6p21.31	rs210142	*BAK1*	[50]	Intronic	1.37
6q25.2	rs2236256	*IPCEF1, OPRM1*	[51]	3′-UTR	1.23
7q31.33	rs17246404	*POT1*	[51]	3′-UTR	1.22
8q22.3	rs2511714	*ODF1*	[51]	Intergenic	1.16
8q24.21	rs2456449	*CASC19*	[45]	Intergenic	1.26
9p21.3	rs1679013	*CDKN2B-AS1*	[44]	Intergenic	1.19
10q23.31	rs4406737	*ACTA2, FAS*	[44]	Intronic	1.27
11p15.5	rs7944004	*C11orf21*	[44]	Intergenic	1.20
11q24.1	rs735665	*GRAMD1B*	[47]	Intergenic	1.45
12q24.13	rs10735079	*OAS3, OAS1*	[48]	Intronic	1.18
15q15.1	rs8024033	*BMF*	[44]	Intergenic	1.22
15q21.3	rs7169431	*RFX7*	[45]	Intergenic	1.36
15q23	rs7176508	*PCAT29*	[47]	Intergenic	1.37
16q24.1	rs391525	*IRF8*	[49]	Intronic	1.82
	rs305061	*IRF8*	[45]	Intergenic	1.22
18q21.32	rs4368253	*PMAIP1*	[44]	Intergenic	1.19
18q21.33	rs4987855	*BCL2*	[44]	3′-UTR	1.47
	rs4987852	*BCL2*	[44]	3′-UTR	1.41
19q13.3	rs11083846	*PRKD2*	[47]	Intronic	1.35

OR odds ratio

[a]OR given is as reported in referenced study

variants identified by GWAS account for approximately 16.5% of the familial risk [43]. Therefore it seems likely that new larger studies or meta-analysis of existing datasets will provide the power to identify further common genetic variants that contribute to CLL risk. Furthermore, it is possible that some of the "missing heritability" may be explained by low-frequency variants (<1%) of intermediate effect size. The advent of next-generation sequencing, which allows high-throughput examination of whole-exome and whole-genome DNA sequences, should extend our capabilities to examine the impact of these germline variants on CLL risk.

Using Genetic Associations to Understand CLL Biology

With more than 30 regions of the genome that play a role in CLL susceptibility now identified, a somewhat clearer picture of the key biological processes involved in CLL development is beginning to emerge. Network-based approaches have revealed that perturbation of apoptosis-related pathways is an important factor in CLL with SNPs close to *BCL2L11*, *BMF*, *BCL2*, *PMAIP1*, and *BAK1* (part of the intrinsic BCL2-regulated apoptosis pathway) as well as *FAS* and *CASP8* (part of the extrinsic death receptor-led pathway)

being robustly associated with CLL risk (Fig. 6.2). It is noteworthy that BH3 mimetics such as Venetoclax [52] inhibit the pro-survival BCL2 proteins, facilitating apoptosis of CLL cells, thus illustrating how GWAS discoveries could prove useful in identifying future CLL treatment targets.

GWAS findings also implicate dysfunctional lymphocyte proliferation and immune response in CLL susceptibility. *IRF4* is a strong candidate for involvement in CLL risk a priori, being a key regulator of lymphocyte development and proliferation. Moreover, *IRF4* expression is involved in the development of CLL [53] and multiple myeloma [54]. Through interaction with transcription factors including PU.1, IRF4 controls the termination of pre-B-cell receptor signaling and promotes the differentiation of pro-B-cells to small B-cells. Furthermore, via BLIMP1 and BCL6, IRF4 controls the transition of memory B-cells, thought to be a precursor cell type for CLL, to plasma cells [55–57]. A model of disease etiology based on the causal variant impairing transition of memory B-cells through reduced IRF4 expression is supported by the association of the risk allele with lower *IRF4* mRNA levels in Epstein–Barr virus (EBV)-transformed lymphocytes [47].

Variation in *IRF8* also represents a strong candidate for the association with CLL risk through its regulation of the α (alpha)- and β (beta)-interferon response. Moreover, *IRF8* is involved in B-cell lineage specification, with IRF8 deficiency skewing development of progenitor cells toward myeloid lineages at the expense of B-cells [58]. Additionally, IRF8 is implicated in immunoglobulin rearrangement and the selection of high-affinity B-cell receptor clones in the germinal center [59, 60]. It has also been shown to negatively regulate the differentiation of plasmablasts, antagonizing the actions of IRF4 [59, 61], and is a mediator of the extrinsic apoptosis pathway [62, 63].

SP140 is the lymphoid-restricted homolog of *SP100* expressed in all mature B-cells and plasma cell lines, as well as some T-cells [64, 65]. SP100 is a major mediator of EBV-encoded nuclear antigen leader protein co-activation, which is important for establishment of latent viral infections and B-cell immortalization [66]. *SP140* expression has also been

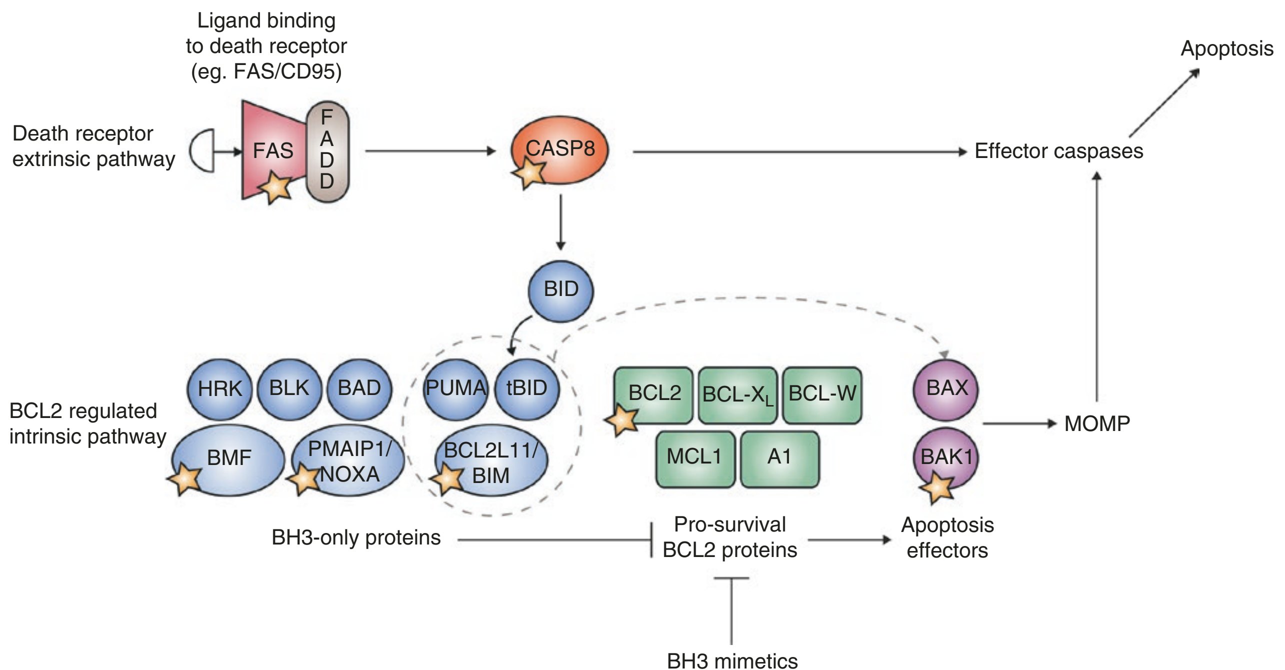

Fig. 6.2 Variation in components of the intrinsic and extrinsic apoptosis pathways is implicated in CLL predisposition. Apoptosis in humans can occur via the intrinsic or extrinsic pathway. In the intrinsic pathway, apoptotic stimuli activate the pro-apoptotic BH3-only proteins which can sequester the pro-survival B-cell CLL/lymphoma 2 (BCL2) family members, thus enabling activation of pro-apoptotic effectors BCL2-associated X protein (BAX) and BCL2-antagonist/killer 1 (BAK1). Some BH3-only proteins (surrounded by dotted line) may also directly activate BAX and BAK1, while therapeutic BH3 mimetics function by inhibiting BCL2 proteins. The pro-apoptosis effectors facilitate mitochondrial outer membrane permeabilization (MOMP), which leads to a cascade of effector caspases that culminates in cell death. The extrinsic apoptosis pathway is activated by binding of a death receptor ligand to its receptor. One such receptor is FAS (also known as CD95). Ligand-receptor engagement leads to CASP8 (caspase 8) activation via the FAS-associated death domain (FADD). Active CASP8 links to the intrinsic pathway by converting the BH3-only protein, BH3 interacting domain death agonist (BID) to truncated BID (tBID), and also feeds into the effector caspases which catalyze cellular destruction. GWAS have identified SNPs near to *BMF*, *PMAIP1*, *BCL2L11*, *BCL2*, *BAK1*, *FAS*, and *CASP8* that are associated with CLL risk, demonstrating that dysfunctional apoptosis is likely to play an important role in disease susceptibility

implicated in host response to immunodeficiency virus type 1 [67]. Another CLL susceptibility locus resides close to *OAS3* and *OAS1*. The *OAS* genes encode enzymes that produce 2′–5′-linked oligoadenylates, which in turn activate the RNaseL system to limit viral replication following infection [68]. It is therefore possible that CLL risk is increased by alterations in the immune response to viral challenge.

LEF1, encoding lymphocyte enhancer factor-1, is a member of the LEF-1/TCF family of transcription factors which acts via the Wnt signaling pathway. LEF1 is vital for the proliferation and survival of B-cells and T-cells and overexpressed in CLL compared to normal mature B-cell subsets [69, 70]. *EOMES* (eomesodermin) plays a role in CD8+ T-cell differentiation [71] and the relative levels of CD8+ T-cells expressing high levels of *EOMES* are critical in maintaining the antiviral T-cell response to chronic infection [72]. SNPs at the *EOMES* locus have also been associated with Hodgkin's lymphoma [73], as well as two autoimmune disorders: multiple sclerosis [74] and rheumatoid arthritis [75].

Mechanisms of Effect of CLL Risk Variants

Although the proximity of CLL risk variants to biologically plausible genes implicates these genes in the etiology of CLL, it remains to be elucidated exactly how the change of a single-nucleotide of DNA sequence can modulate an individual's susceptibility to CLL. Only one of the published SNPs is a missense variant. SNP rs757978 results in the substitution of threonine for isoleucine at amino acid 260 in *FARP2* (FERM, RhoGEF, and Pleckstrin Domain Protein 2) and is predicted to be deleterious by the SIFT algorithm [76].

The majority of published CLL GWAS SNPs reside within noncoding regions of the genome and it is likely that they exert their effects on CLL risk through *cis*-regulation of nearby genes. Indeed, correlations between SNP genotype and mRNA levels have been shown for a number of the risk SNPs including rs872071 and *IRF4*; rs13397985 and *SP140*; rs210134 and *BAK1;* rs10936599 and *TERC*; as well as rs10735079 and *OAS1/2/3* [77–79].

The mechanisms by which these SNPs modulate gene expression might involve mediation of chromatin accessibility, differential transcription factor binding, and/or formation of looping chromatin interactions. Such effects have recently been described for SNPs associated with type 2 diabetes [80], prostate cancer [81], and neuroblastoma [82] although perhaps the archetypal model for a noncoding variant mediating a long-range regulatory effect is demonstrated by previous work on the 8q24.21 interval in colorectal cancer. In this instance the associated SNP, rs6983267, resides 335 kb upstream of *MYC*; however the risk locus is characterized by enhancer-related histone modifications and can form chromatin loop to the *MYC* promoter and the risk variant is shown to increase binding of the transcription factor TCF4 [83–85]. Given that 8q24.21 also harbors a risk variant for CLL, which resides some 565 kb upstream of *MYC* [45], it is appealing to predict a similar mode of action. This is especially relevant because MYC and IRF4 form an auto-regulatory loop during B-cell activation [54].

Monoclonal B-cell Lymphocytosis as a Precursor Condition

The recognition that common variants influence the risk of CLL raises the possibility that, while clinically diagnosed CLL may be uncommon in the population, susceptibility may be far more common. Intriguingly this assertion is supported by the observation that CLL-phenotype B-cells (CD5+, CD23+, CD20low, sIgMlow) of monoclonal B-cell lymphocytosis (MBL) are detectable in ~3% of adults in the general population [86] and that they are essentially indistinguishable from CLL B-cells in terms of chromosomal abnormalities and *IGHV* mutation status.

The recent report that MBL develops into CLL at a rate of 1.1% per year provides direct evidence that MBL is a precursor lesion for CLL [87]. These data coupled with the observation that approximately 10% of relatives of familial CLL patients have MBL support the assertion that MBL is a surrogate marker for genetic predisposition. In an evaluation of 10 CLL risk variants in 419 MBL cases and 1753 controls, six of the risk SNPs were also significantly associated with MBL, thus providing further evidence for MBL being a precursor condition to CLL [88].

Links Between Germline Variants and the Tumor

A small number of GWAS SNPs have been associated with clinical features of CLL. For example, the *IRF8* variant rs305061 was associated with IGHV mutational status, with the risk allele correlating with poor prognosis unmutated CLL [45]. In a separate study of 840 CLL cases, carriers of the risk allele of the *IRF4* 3′UTR variant rs872071 had a significantly shorter treatment-free survival time than non-carriers [89].

Recent next-generation sequencing efforts of tumor DNA from CLL patients [90–92] have identified recurrent somatic mutations in two genes (*POT1* and *IRF4)* which also harbor common germline predisposition variants. As studies of the tumor DNA are extended, it will be of interest to note whether further overlap with germline risk factors is identified or to determine whether germline factors might predispose to particular somatic changes.

Conclusions and Reflections

Our knowledge of the role of germline genetic factors in CLL predisposition is rapidly progressing. It is now well established that the disease is characterized by having amongst the highest familial risks of any malignancy. Moreover, the observation that MBL represents a progenitor lesion offers considerable opportunities for understanding the key events in the development of CLL.

Using the GWAS approach, over 30 susceptibility loci for CLL have been identified. Further investigation of these regions to identify the causal genetic variants is likely to yield mechanistic insights into the biological basis of CLL. Building on our current understanding that impaired apoptotic processes and a dysfunctional immune response are important in disease predisposition could also help identify potential therapeutic targets.

Despite the success of GWAS, a substantial proportion of the familial risk of CLL remains unexplained. In part this may be accounted for by the presence of low-frequency variants of moderate effect size, too rare to be captured by GWAS but with lower effect sizes than would be expected in Mendelian disease. To this end, utilization of whole-exome and whole-genome sequencing strategies could help to identify additional genes involved in CLL predisposition and further advance our knowledge of this complex disease.

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Morphology and Immunophenotype of Chronic Lymphocytic Leukemia

7

Mir Basharath Alikhan and Girish Venkataraman

Introduction

Chronic lymphocytic leukemia (CLL) is a low-grade, mature B-cell lymphoproliferative disorder (LPD) characterized by proliferation of small lymphocytes in the bone marrow and peripheral blood, often with extramedullary involvement in lymphoid tissues, such as lymph nodes and spleen [1]. The most common mature B-cell lymphoma, particularly in Western countries, CLL was first clinically described in 1845 and morphologically characterized by Minot and Isaacs in 1924 [2, 3]. Since these initial descriptions, the diagnosis of CLL is now often made by a combination of characteristic morphologic and immunophenotypic features. While the diagnosis of CLL is often straightforward based on these criteria, distinction from other mature B-cell lymphomas or reactive conditions may sometimes be challenging. Monoclonal B-cell lymphocytosis is a related entity now thought to represent the precursor to overt CLL, and consists of "low-count" and "high-count" variants; the two have significant clinical and prognostic differences [4]. Recent advances in the molecular genetics of CLL necessitate further ancillary studies to ensure timely and adequate treatment, including targeted therapies, for patients requiring intervention. Although CLL is best characterized as an indolent neoplasm, progression to an aggressive lymphoma, most often either large-cell transformation or less commonly Hodgkin lymphoma transformation, occurs in 1–12% of patients [5–7]. Determination of *IGHV* mutation status, detection of certain chromosome aberrations, and positivity for immunohistochemical markers (such as CD38 and ZAP-70) can help to prognosticate patients and guide therapy.

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Definitions

CLL is a neoplasm of mature B-cells that primarily affects the bone marrow and peripheral blood, and often shows involvement of extramedullary tissues such as lymph node and spleen. According to the World Health Organization (WHO) classification criteria based on the 2008 International Workshop on Chronic Lymphocytic leukemia (IWCLL), the diagnosis of CLL can only be made in the presence of an absolute B-cell monocytosis of ≥5000 B-lymphocytes/μL in the peripheral blood for at least 3 months [1, 8]. Although most cases of CLL show a characteristic immunophenotype (B-cells with CD5 and CD23 co-expression), these features are not always present and thus not strictly required for the diagnosis [9].

The term "small lymphocytic lymphoma (SLL)" is given to neoplasms which lack a significant leukemic component and show a predominant extramedullary disease distribution, often in lymph nodes and spleen. Patients present with lymphadenopathy in the absence of any cytopenias or significant lymphocytosis. In these cases, the degree of lymphocytosis does not meet the criteria of CLL (e.g., absolute B-lymphocytosis is <5000 B-cells/μL).

On the other hand, patients with a smaller population of circulating monoclonal B-cells that do not meet the criteria for CLL and are asymptomatic (i.e., without lymphadenopathy, cytopenias, or autoimmune disease) are classified as having monoclonal B-cell lymphocytosis (MBL) [4, 10] (see Table 7.1 for timeline of important studies in MBL). This is a heterogeneous entity wherein otherwise healthy patients are found to have small populations of clonal B-cells in the peripheral blood. The term "lymphocytosis" does not refer to overall absolute lymphocytosis, but only to the monoclonal B-cells. Indeed, patients with MBL may have normal white cell peripheral blood counts. These populations of clonal B-cells are essentially absent in healthy individuals.

MBL is subclassified into three immunophenotypic categories and two clinical subtypes. With regard to the immunophenotypic subtypes of MBL, the vast majority have a typical

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Table 7.1 Timeline summary of major MBL-related studies leading up to the WHO 2016 revision

Author (year)	Comment
Marti G (2005) [10]	Publication of consensus guidelines of MBL: definition of absolute B-cell count (ABCC) <5000/μL in the absence of signs and symptoms of lymphoproliferative disorder or autoimmune disease
Swerdlow (2008) [11]; Hallek M (2008) [8]	Adoption of 2005 consensus guidelines by WHO and International Workshop on CLL
Rawstron AC (2008) [12]	Identification that cases with ABCC <1900/μL had a very low rate of progression to CLL
Rawstron AC (2010) [13]	Meta-analysis noting bimodal distribution of clonal B-cells within MBL with one peak between 0.1 and 10 B-cells/μL and another at >500/μL
Gibson (2011) [14] & Ghia (2011) [15]	Recognition of "nodal counterpart" of MBL
Vardia A (2013) [16]	Closer immunogenetic relationship of HC-MBL to Rai stage 0 CLL based on similar somatic hypermutation and BCR stereotypy
Swerdlow (2016) [17]	WHO 2016 revision emphasizing the need for closer follow-up of HC-MBL cases; disallows ability to diagnose CLL with ABCC <5000/μL with cytopenias in the absence of clinical signs or symptoms

CLL-type immunoprofile, with expression of B-cell marker CD19, co-expression of CD5 and CD23, and dim expression of CD20 and surface light and heavy chain [18]. Other, less common subtypes include the non-CLL phenotype, without expression of CD5, and atypical CLL phenotype, with co-expression of CD5 but with strong expression of CD20 and/or negative or low expression of CD23. Mantle cell lymphoma (MCL) should be excluded in those with atypical CLL phenotype while those with the non-CLL profile may be related to marginal zone or lymphoplasmacytic lymphomas [19].

More clinically important is the distinction between high-count MBL (HC-MBL) and low-count MBL (LC-MBL), based on the concentration of circulating clonal B-cells. The cutoff most widely applied to distinguish the two subtypes is 500 clonal B-cells/μL. LC-MBL is generally detected in asymptomatic patients without lymphocytosis, often as an incidental finding or in the setting of population-based studies, using high-sensitivity flow cytometry techniques. Employing eight-color flow cytometry analysis in population studies showed that CLL-like clonal B-cells are present in up to 12% of the general population, more often in the elderly [20]. In contrast, patients with HC-MBL present with lymphocytosis and are recognized in a clinical setting.

The biologic relationship between HC-MBL and CLL is significant, as 1–4% of patients with HC-MBL per year go on to develop overt CLL, highlighting the importance of lifelong follow-up of lymphocytosis [12, 21] as well as the notion that HC-MBL is an obligate precursor of CLL. LC-MBL, conversely, does not require follow-up as the risk of progression is quite low and is not considered a pre-leukemic condition [16].

Another line of evidence that supports the relationship between HC-MBL and CLL is the analysis of immunoglobulin gene repertoires. LC-MBL often shows rearrangement of the *IGHV4-59/61* gene which is rarely observed in CLL. In contrast, HC-MBL shows a repertoire more similar to CLL, such as expression of *IGHV3-23* and *IGHV4-34* [16]. Additionally, mutational analysis using massive parallel deep sequencing found similar acquired genetic mutations between HC-MBL and CLL (such as *NOTCH* and *Wnt*) [22] and was unable to distinguish between HC-MBL and Rai stage 0 CLL. Factors related to progression from HC-MBL to CLL remain largely elusive, with some suggesting that noncoding mRNAs, particularly microRNAs such as miR-155, have a role to play [23].

Finally, it is important to note that although most MBL are CLL-like with regard to immunophenotype, non-CLL forms exist. In these cases, the diagnostic considerations include mantle cell lymphoma (in CD5+, CD23− cases; atypical CLL-like) or marginal zone lymphoma (in CD5− cases; non-CLL) [24]. Appropriate ancillary studies should be carried out to exclude these entities.

Epidemiology and Clinical Aspects

CLL is the most common adult mature B-cell leukemia in the Western world, where it accounts for 30% of all leukemias in this population. The incidence increases in the elderly and the median age at diagnosis is about 70 years [25]. Similarly, the incidence of MBL also increases with advanced age, paralleling CLL. Familial cases of CLL have also been described [26]. It is estimated that in 2016, the about 19,000 new cases of CLL will be diagnosed in the United States and about 5000 patients will die of the disease [25]. SLL accounts for about 7% of all non-Hodgkin lymphomas and also tends to occur in elderly males.

Due to more accurate and sensitive diagnostic techniques, the vast majority of CLL patients (up to 70%) are asymptomatic at presentation [27, 28], and the disease is often detected as an incidental finding on routine complete blood counts, which, in most cases, will show preserved hematopoietic cell lineages. Symptomatic patients may show cytopenias, which is most often due to infiltration of the bone marrow by leukemic cells. Autoimmune cytopenias are fairly frequently described in CLL and may affect all three cell lines. Anemia may also be attributed to immune-mediated destruction of red blood cells or red cell precursors (pure red cell aplasia). Although multiple mechanisms are postulated, one line of evidence suggests that CLL B-cells may act as antigen-processing cells which take up red cell antigens, and induce a secondary T-cell activation which in

turn leads to activation of normal B-cells resulting in red cell antibody production and consequent destruction of red cells [29]. Hypogammaglobulinemia may also be present at the time of diagnosis in up to half of cases.

Extramedullary disease in CLL/SLL comes to clinical attention most often as lymphadenopathy and, less commonly, splenomegaly. Not uncommonly, it is diagnosed on lymphadenectomy specimens excised for other tumors, such as carcinomas [30]. Lymphocytic infiltrates can be found in a variety of other anatomic sites, such as gastrointestinal tract, kidney, liver [31], and skin. However, with the exception of possibly the latter two sites, these often do not manifest clinically and are detected as part of routine workup. Cutaneous involvement by CLL can present at localized disease or generalized erythematous papules, plaques, nodules, or tumors [32]. Rare sites of involvement include Waldeyer's ring, thyroid, lung, gallbladder, prostate, central nervous system, leptomeninges, serosal surfaces, and other sites [33–38].

Cell of Origin and Pathogenesis

The understanding of CLL leukemogenesis has undergone considerable modifications in the past few years. Most now agree that CLL derives from an antigen-experienced B-cell that can be of two general subtypes: either with somatic mutations in the variable genes of the immunoglobulin heavy-chain gene (*IGHV*), termed "mutated CLL" which are derived from CD5+/CD27+ post-germinal center B-cells, or with germline configuration (or low-level somatic hypermutation) of the gene, referred to as unmutated CLL which in turn are derived from CD5+/CD27− mature B-cells.

At a molecular level, unmutated CLL is defined as having at least 98% sequence homology to the germline [39]. Mutated CLL characterizes the majority of cases. In both subtypes, initial inducing mutations in the B-cell provide a growth advantage [40]. Unmutated CLL cells express surface immunoglobulin receptors with relatively more intact/functional BCR signaling pathway that can be activated by multiple antigens such as auto-antigens and carbohydrate components of microbes. Repetitive stimulation of the induced B-cells by these antigens results in clonal proliferation and leukemia. In contrast, CLL cells with mutated *IGHV* genes do not have the polyreactivity seen in unmutated CLL cells, and their ability to proliferate is relatively diminished compared to the latter. In some cases, anergy develops due to excessive B-cell receptor stimulation, leading to compromise of antigen binding and subsequent apoptosis.

However, changes in the B-cell receptor signaling pathway promote expansion of the leukemic clone and development of CLL. In particular, CLL cells overexpress Toll-like receptor 9 (TLR9), an innate immune receptor that responds to bacterial unmethylated cytosine guanine dinucleotide (CpG) oligodeoxynucleotides (ODNs) [41]. When stimulated, TLR9, through coregulation with MYD88, induces the NF-κB pathway, resulting in apoptosis. Some have suggested that this pathway may be exploited through TLR9 stimulators, such as CpG ODNs [42]. Zeta-associated protein 70 (ZAP70, see below) is often seen in unmutated CLL. In such cases, activation of TLR9 leads to ZAP70-dependent activation of spleen tyrosine kinase (Syk). This results in the degradation of proapoptotic protein Bim, leading to survival of the CLL clone. Additionally, activation of Syk leads to downstream production of autoreactive IgM, which binds the B-cell receptor complex and, in an autocrine manner, causes a continual positive feedback loop [43]. This model may explain why unmutated status of CLL has a poor prognosis compared to the mutated type, as the former is subject to constant antigenic stimulation and production of leukemic subclones [44] as well as increased tumor cell survival. Interestingly, up to 30% of unmutated CLL show similar B-cell receptors, suggesting that a unique set of antigens is responsible for clonal expansion. One may speculate that stimulation at the pre-CLL stage by antigens such as an environmental agent, i.e., a virus or commensal bacteria, or specific autoantigen is responsible for leukemic proliferation [45–47].

Additional lines of evidence that supports the derivation of CLL cells from activated B-cells as opposed to naïve B-cells as was previously thought include similar gene expression profiling data to memory B-cells, presence of mRNA for various cytokines, and expression of memory cell markers such as CD27, regardless of mutational status [48, 49]. The activated B-cell may arise from the marginal zone of follicles or from germinal centers. Those arising from germinal centers require T-cell activation and mutation of *IGHV* genes. In contrast, CLL cells from the marginal zones may or may not require T-cell activation, and only a subset show mutation of *IGHV* genes.

Although the putative normal counterpart of the CLL cell is thought to be an antigen-experienced memory B-cell as outlined above, some studies have shown that the earliest genetic changes leading to CLL can be found in hematopoietic stem cell (HSC). In particular, HSCs from patient will CLL were successfully engrafted into immunodeficient mice and resulted in clonal B-cell lymphoproliferations [50]. The data suggest that these HSCs are primed towards the B-cell lineage and subsequent clonal proliferation.

Morphology of CLL/SLL

Peripheral Blood

Involvement of peripheral blood is required in the diagnosis of CLL and is often the initial mode of diagnosis. As mentioned above, there is absolute lymphocytosis with monoclonal B-cells exceeding a concentration of 5000/μL. The

overall white cell count may be normal or elevated. Patients may be anemic, which is most often attributed to leukemic infiltration of the bone marrow, although autoimmune erythrocyte destruction is also common at the time of diagnosis.

The circulating leukemic cells of CLL are often monotonous, small- to medium-sized lymphocytes with fairly round and even nuclear membranes, scant agranular, basophilic cytoplasm, inconspicuous nucleoli, and exaggerated clumping of nuclear chromatin. This latter feature is often characterized as a "cracked mud," "chocolate chip cookie," or "soccer ball" appearance of the nuclei on well-stained smears (Fig. 7.1). The cytoplasm may contain vacuoles or crystals and can occasionally be more abundant. Cytoplasmic inclusions containing immunoglobulin have also been described [51]. Smudge cells (Fig. 7.1) are often observed due to the tendency of leukemic cells to be easily disrupted during smear preparation. This is an important clue to the diagnosis of CLL as this feature is lacking or rare in most other B-cell leukemias when analyzing well-prepared smears. The addition of albumin to the peripheral blood can prevent the disruption of leukemic cells and the presence of smudge cells on the smear [52].

Cells with more abundant cytoplasm and a prominent single nucleolus are termed *prolymphocytes* and often constitute less than 10% of the total leukemic cells (Fig. 7.1). If they predominate, the diagnosis of B-cell prolymphocytic leukemia (B-PLL) should be considered, which is defined as a mature B-cell leukemia with greater than 55% prolymphocytes. A patient diagnosed with typical CLL who is found to have rising numbers of prolymphocytes on follow-up smears may represent transformation to a higher grade leukemia. Other patients may present with large numbers of prolym-

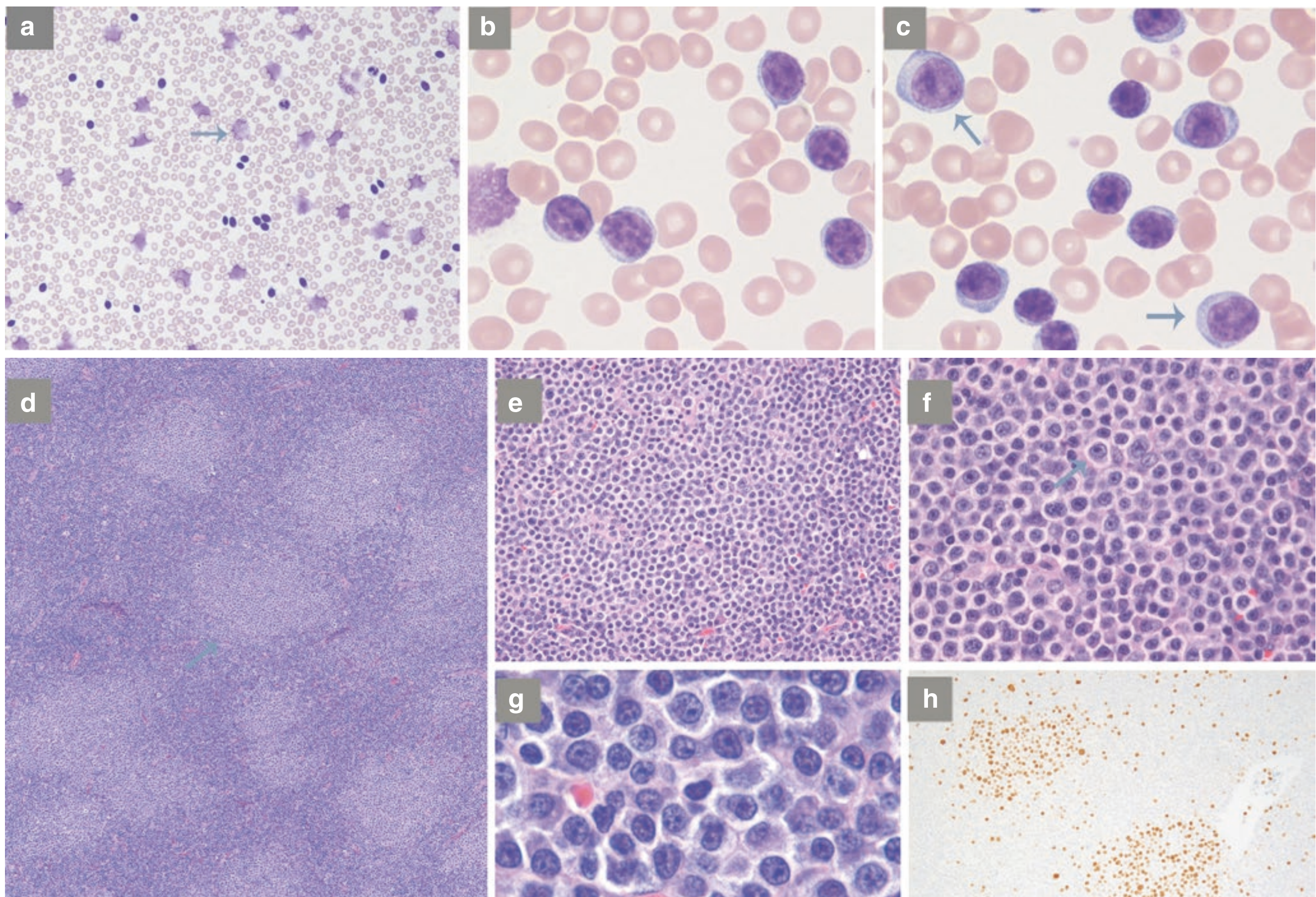

Fig. 7.1 CLL morphology in peripheral blood and lymph nodes: (**a**) Peripheral blood with absolute lymphocytosis and numerous smudge cells (*arrow*) which are sheared CLL cells with fragile cytoplasmic membranes. This smudging can be prevented by incubating the cells with bovine albumin before preparing the smear. (**b**) High power of CLL cells in peripheral blood demonstrated small CLL cells with typical "cracked-mud" chromatin and inconspicuous nucleoli. (**c**) CLL with trisomy 12 demonstrating increased number of larger cells with prominent nucleoli consistent with prolymphocytes. (**d**) Low-power histology of CLL with diffuse sheets of small lymphoid cells and associated nodular clusters of pale areas called "growth centers/proliferation centers." CLL is probably unique in being the only lymphoma exhibiting such proliferation. (**e, f**) High-power views of a growth center with scattered prolymphocytes and paraimmunoblasts that are larger cells with prominent nucleoli (H&E stain). (**g**) High-power view of CLL cells in lymph node demonstrating clumped nuclear chromatin and inconspicuous nucleoli. (**h**) Growth centers with proliferating cells (Ki-67 immunostain)

phocytes in the blood that remain stable throughout the disease course. A recent study found that increased circulating prolymphocytes at presentation with distinct immunohistochemical and genetic variation from classic CLL was an independent adverse prognostic factor with frequent progressive disease to Richter transformation and shorter overall survival [53].

Slight differences in the classic morphology may be observed. The nuclear contours can sometimes be irregular and mildly indented and/or the lymphoid cells are larger. In cases of typical CLL, these account for less than 2% of the leukemic cells. When they are more abundant, the diagnosis of *atypical* CLL should be considered (see below). In such cases, cytogenetic analysis should be performed as specific findings have been linked with atypical morphology, particularly trisomy 12, which can present with slightly higher numbers of prolymphocytes or CLL cells with atypical morphology (Fig. 7.1) [39, 54–56].

Plasmacytoid differentiation can also be observed in atypical CLL and has morphologic overlap with lymphoplasmacytic lymphoma. Patients may present with an IgM paraprotein, but levels often do not exceed 3 g/dL. The morphologic features often lead to diagnostic difficulty, but the presence of prolymphocytes/paraimmunoblasts, proliferation centers, and typical CLL/SLL immunophenotype aids in the diagnosis of CLL/SLL over lymphoplasmacytic lymphoma in such cases [57]. Some have suggested a possible association with del(7)(q32) [58].

Uncommonly, Reed-Sternberg-like cells can be observed in otherwise typical cases of CLL/SLL, characterized by the presence of rare or occasionally large, pleomorphic cells. Only if a distinct region of the biopsy has typical Hodgkin inflammatory background can the diagnosis of transformation to Hodgkin lymphoma be rendered [59]; sparse presence of R-S-like cells in otherwise extensive small lymphocytic proliferation is not diagnostic of transformation. Epstein-Barr virus is often detected in these cases.

A recent study suggests that increased circulating monocyte-derived cells promote CLL survival through cell-cell interactions and secreted factors and thus worse patient outcomes. Indeed, increased monocytes were found to be an adverse prognostic factor leading to decreased time to first therapy and overall survival [60].

In all cases of atypical CLL, cytogenetic analysis should be performed, as studies have consistently shown adverse prognosis, more severe cytopenias, worse lymphocytosis at presentation, and more rapid disease progression [61]. Such cases can also show immunohistochemical differences from classic CLL and the results can overlap with other B-cell lymphoproliferative disorders such as mantle cell lymphoma, lymphoplasmacytic lymphoma, and marginal zone lymphoma [62].

Bone Marrow

Although the bone marrow is invariably involved in virtually all cases of CLL, bone marrow biopsy examination is not required to render the diagnosis. However, the biopsy may be helpful in assessing the degree of involvement and aid in correlation with peripheral blood cell counts in cases presenting with cytopenias. Leukemic cells in the marrow aspirate are morphologically similar to those in the peripheral blood (Fig. 7.2) and often comprise greater than 30% of all nucleated cells. However, the percentage of CLL cells may differ, due to the multifocal nature of the disease in the marrow. In view of this, examination of an adequate bone marrow trephine biopsy (greater than 10 mm in length, but preferably up to 20 mm, taken from the posterior iliac crest) provides more useful information with regard to the extent of involvement [63]. Additionally, the histologic pattern of involvement can aid in the diagnosis of CLL and help exclude other lymphomas in the differential.

Various patterns of CLL infiltration may be observed, and multiple patterns can be observed within the same bone marrow biopsy. The most common architectural pattern seen is multifocal, interstitial nodular involvement (Fig. 7.2). Importantly, the nodules are non-paratrabecular, in contrast to bone marrow infiltrates of follicular lymphoma, which characteristically show paratrabecular localization. On low-power examination of the biopsy, the nodular interstitial infiltrates are readily identifiable due to their darker appearance compared to the surrounding hematopoietic marrow. On higher magnification, the cells are small with round nuclei, inconspicuous nucleoli, and a clumped chromatin pattern. The edges of the nodules may show diffusion of the leukemic cells into the marrow interstitium. Proliferation centers that harbor prolymphocytoid cells are common in lymph nodes involved by CLL/SLL, but not usually observed in the marrow. The presence of germinal centers (interfollicular pattern) is quite rare; their presence should prompt exclusion of other lymphomas [64].

Other common patterns include a more diffuse interstitial infiltrate wherein the leukemic cells are in close association with and interspersed between the normal hematopoietic marrow elements with effacement of the overall marrow architecture. These areas are more difficult to identify on low-power examination than the nodular infiltrates. However, they often impart a slightly darker appearance than the surrounding normal marrow elements. Less commonly, solid growth of leukemic cells replacing the marrow elements can be seen. In such cases, peripheral cytopenias are expected, which can predict a more aggressive disease and worse overall outcome. Some studies have demonstrated that a solid growth pattern is linked with expression of the 70-kDa zeta-associated protein (ZAP-70) and unmutated status of *IGHV*, which is thought to confer a worse prognosis [65, 66].

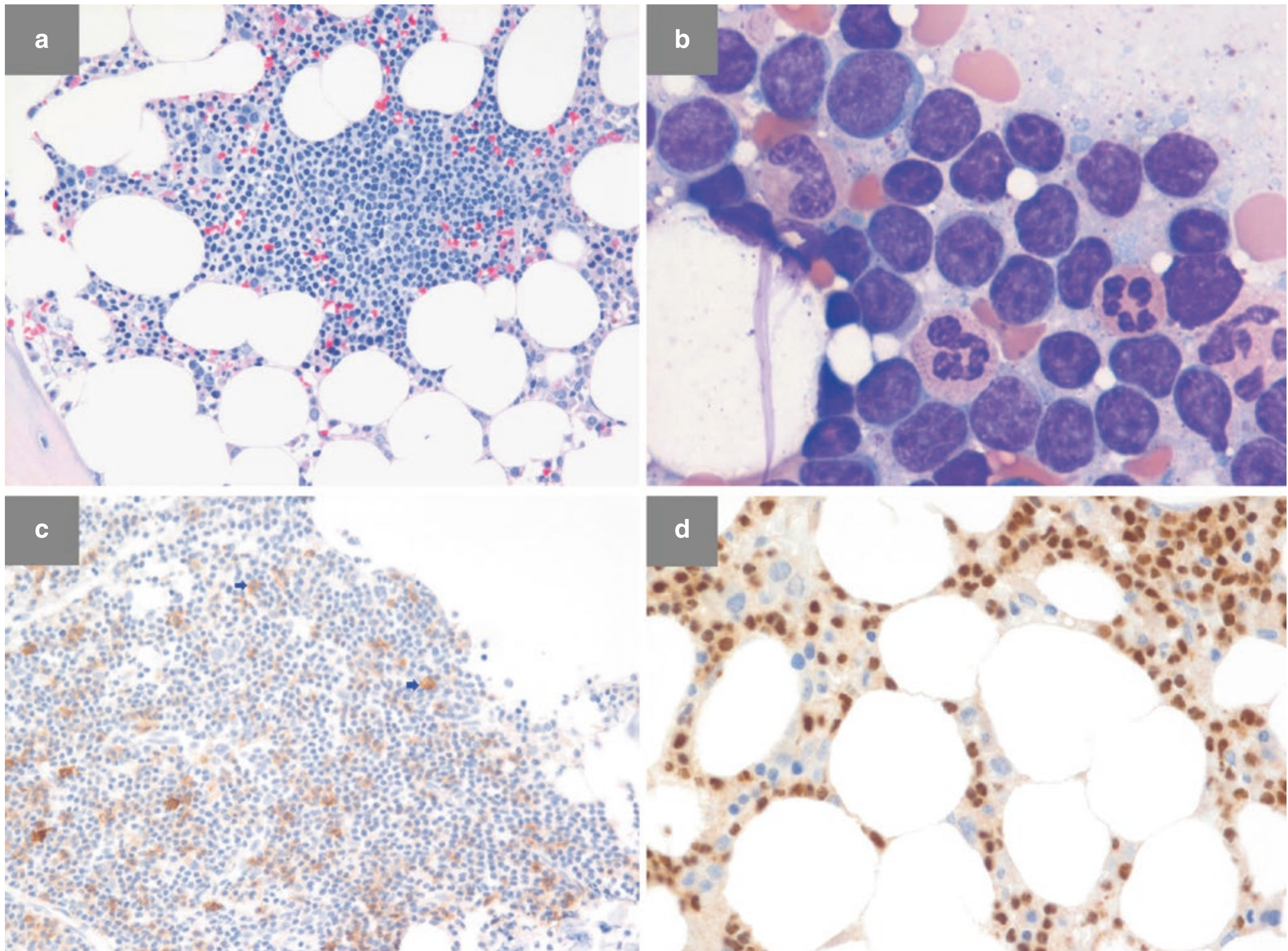

Fig. 7.2 Bone marrow in CLL: (**a**) Nodular infiltrate of small lymphoid cells of CLL involving the interstitial areas of the marrow. (**b**) High-power view of CLL cells in the marrow aspirate. (**c**) CD20 immunostain in a CLL with very dim expression in a subset of the B-cells (*arrow*). CD20 may also be downregulated after rituximab-containing regimens such as fludarabine, cyclophosphamide, and rituximab (FCR). In such cases, Pax5 may be more helpful. (**d**) Pax5 immunostain highlights prominent interstitial infiltrate of CLL cells

Marrow involvement can be detected in most MBL cases and with three different patterns described (focal interstial, nodular and diffuse interstitial) in one study examining 26 MBL cases (CLL-like, atypical CLL-like, and non-MBL types). In this study focal interstitial pattern was the most common pattern observed in most CLL-like and atypical CLL-like MBL. Uncommonly, the bone marrow may be the first detected site of involvement by transformed CLL, showing enlarged, atypical lymphoid cells that differ from the usual small cells of classic CLL [67].

Marrow biopsy is often performed to assess the underlying cause of cytopenias which may be secondary to diffuse involvement of the marrow by leukemic cells or autoimmune destruction of one or more cell lineages. The presence of erythroid hyperplasia in an anemic patient may suggest peripheral red cell destruction due to autoimmune disease, which is not an uncommon presentation in CLL patients [68]. Anemia without a marrow response can indicate the rare instance of pure red cell aplasia [69]. In turn, red cell aplasia can result as a result of direct immune destruction by activated B-cells or evolve as a result of secondary T-large granular lymphocyte expansion in the context of CLL.

Another advantage of assessing the bone marrow biopsy is the ability to perform immunohistochemical stains, particularly when fresh cells are not available for flow cytometric analysis. The immunophenotype by immunohistochemistry would be similar to results of flow cytometry (see below). However, some important caveats when assessing immunohistochemistry results include careful evaluation of CD20 staining. As in flow cytometry, CD20 expression can be dimmer than the surrounding normal B-cells (Fig. 7.2). In patients that have undergone anti-CD20 immunotherapy, CD20 expression can be lost and hence may not be reliable in picking up the CLL population. In these cases, staining with PAX5 (Fig. 7.2) can be helpful to determine B-cell lineage.

Lymph Node

The predominant pattern of lymph node involvement by CLL/SLL is diffuse effacement of the nodal architecture. The lymph node follicles and sinuses are often obliterated, and the neoplastic cells can extend into the surrounding adipose tissue. The predominant neoplastic cell is small to medium sized with clumped chromatin, inconspicuous nucleoli, and scant cytoplasm (Fig. 7.1), imparting a dark appearance to most of the affected lymph node. The nucleus is most often round, but can have some irregularity or cleaving which may cause diagnostic confusion with follicular lymphoma or mantle cell lymphoma.

Characteristically, proliferation centers (so-called pseudofollicles) are present throughout the node as part of the diffuse architectural effacement and are characterized by paler staining areas compared to the darker zones of the remainder of the neoplasm (Fig. 7.1). The lighter staining is due to involvement of the proliferation centers by larger lymphoid cells: prolymphocytes and paraimmunoblasts (see below and Fig. 7.1). Prolymphocytes in the lymph node are medium-sized cells with more abundant cytoplasm and a more prominent nucleolus compared to the predominant CLL cells. Paraimmunoblasts are larger, with a wider rim of cytoplasm, dispersed chromatin, and a distinct central eosinophilic nucleolus.

The more abundant cytoplasm of these cells causes separation between the tumor nuclei and imparts the lighter appearance. The contrast can be better appreciated at low-power light microscopy if the light is dimmed. Proliferation centers can also be assessed using a marker of DNA synthesis, such as Ki-67, which would be high in the proliferation centers and virtually negative in the remainder of the lymphoma (Fig. 7.1). If such areas dominate the lymph node histology, concern for high-grade transformation to a large-cell lymphoma should be considered.

Proliferation centers can mimic germinal centers of a normal follicle. However, the absence of a distinct mantle zone and apoptotic debris can be histologic clues of a proliferation center rather than a germinal center. Additionally, immunohistochemical study would show that the cells of proliferation centers are CD10−, Bcl6−, and Bcl2+, in contrast to normal germinal centers [70]. Rarely proliferation centers may also express cyclin D1 but there is no underlying *IgH/CCND1* translocation in these cases [71].

Variant patterns of lymph node involvement are less common and include an interfollicular architecture where the neoplastic cells surround remnant reactive lymph node follicles [72]. A perifollicular pattern is also described. These may lead to diagnostic confusion with follicular lymphoma, marginal zone lymphoma, or mantle cell lymphoma. Helpful features in the diagnosis of CLL/SLL include presence of proliferation centers, absence of cyclin D1 immunostaining, and characteristic immunoprofile (see below). As mentioned above, Reed-Sternberg-like cells can be observed in cases of CLL/SLL. If rare or occasional, they are not considered to represent transformation to Hodgkin lymphoma (HL). However, if more abundant and present with the typical milieu seen in HL, the concern for transformation is much higher.

Spleen

Splenic involvement by CLL is almost always present as part of the generalized disease. However, the degree of involvement is variable. Spleens are almost always enlarged, sometimes to massive weights, but the capsule is usually not compromised [73]. On gross examination, there is a miliary pattern of involvement, with 0.2–1.5 cm micronodules present throughout the cut surface. Histologically, the white pulp is replaced and expanded by proliferation of the small leukemic cells of CLL, similar to the predominant cells seen in the lymph nodes. In contrast to the lymph node morphology, proliferation centers with prolymphocytes and paraimmunoblasts are not usually observed. The tumor often infiltrates into the red pulp cords and sinuses and will extend along the periarteriolar lymphoid sheaths and splenic trabeculae [74, 75].

Other Organs

Although the liver is commonly involved in CLL, clinically apparent hepatic dysfunction is not common [76]. The leukemic cells often infiltrate the portal tracts and less commonly the sinuses can also be involved. The neoplasm can be mistaken for inflammatory infiltrates of hepatitis, for example. However, the monotonous nature of the lymphoid cells and characteristic immunophenotype can help to distinguish the two entities.

Cutaneous involvement can present in a variety for different clinical forms, including plaques, nodules, or tumors. Histologically, the dermis shows leukemic infiltrates that tend to be localized around vascular and adnexal structures [32].

The gastrointestinal tract, central nervous system, and other organs can also be involved, albeit rarely, and are often seen only on autopsy specimens as “case reports” in the literature [77–79].

Immunophenotype of CLL

Immunophenotyping of CLL is one of the most essential aspects of diagnosis and prognosis of the disease, allowing for differentiation between reactive conditions and other lymphoproliferative disorders as well as assessment of adverse prognostic markers. The most useful, rapid, and efficient method of immunophenotyping is multicolor

flow cytometry, which allows for multiparameter testing with multiple antibodies, and facilitates determination of the strength of antigen expression. Most tissues can be submitted for flow cytometry, the easiest being peripheral blood, but also bone marrow aspirates and freshly procured solid tissues such as lymph nodes, following disaggregation. Immunophenotyping on solid, fixed tissues with immunohistochemistry can also be useful in cases when fresh tissue or peripheral blood is not available for analysis.

In the typical form of CLL, neoplastic cells show moderate expression of CD19 and the nuclear B-cell transcription factor PAX5, supporting a B-lymphocytes lineage [80]. Other B-cell antigens are characteristically downregulated (Fig. 7.3) and show weak/dim expression, such as CD20, CD79a/b, and surface immunoglobulin (Fig. 7.3), which is usually IgD with or without IgM. Expression of other immunoglobulin heavy chains (IgG or IgA) is uncommon. This low expression of CD20 and Ig is quite unique to CLL and is an important differentiating clue between this and other B-cell lymphoproliferative disorders [8, 28]. Mechanistically, the low sIg levels in CLL are thought to result from altered glycosylation and folding of the CD79a and μ chains although the BCR signaling remains intact [81].

Another important marker is the co-expression of CD5, a T-cell antigen (Fig. 7.3). Compared to the background T-cells, CD5 expression is slightly weaker. CD5+ B-cells can be found normally in the mantle zones of secondary follicles. Additionally, regenerating naïve B-cell can also express CD5 [82]. The co-expression of CD5 can also be observed in mantle cell lymphoma. However, MCL does not show the weak expression of most B-cell markers as does CLL, and often does not express CD23. Immunohistochemistry for cyclin D1 is present in most cases of MCL, in contrast to typical cases of CLL.

CD23 is a low-affinity receptor for IgE and an adhesion molecule that is expressed by naïve B-cells. It is normally

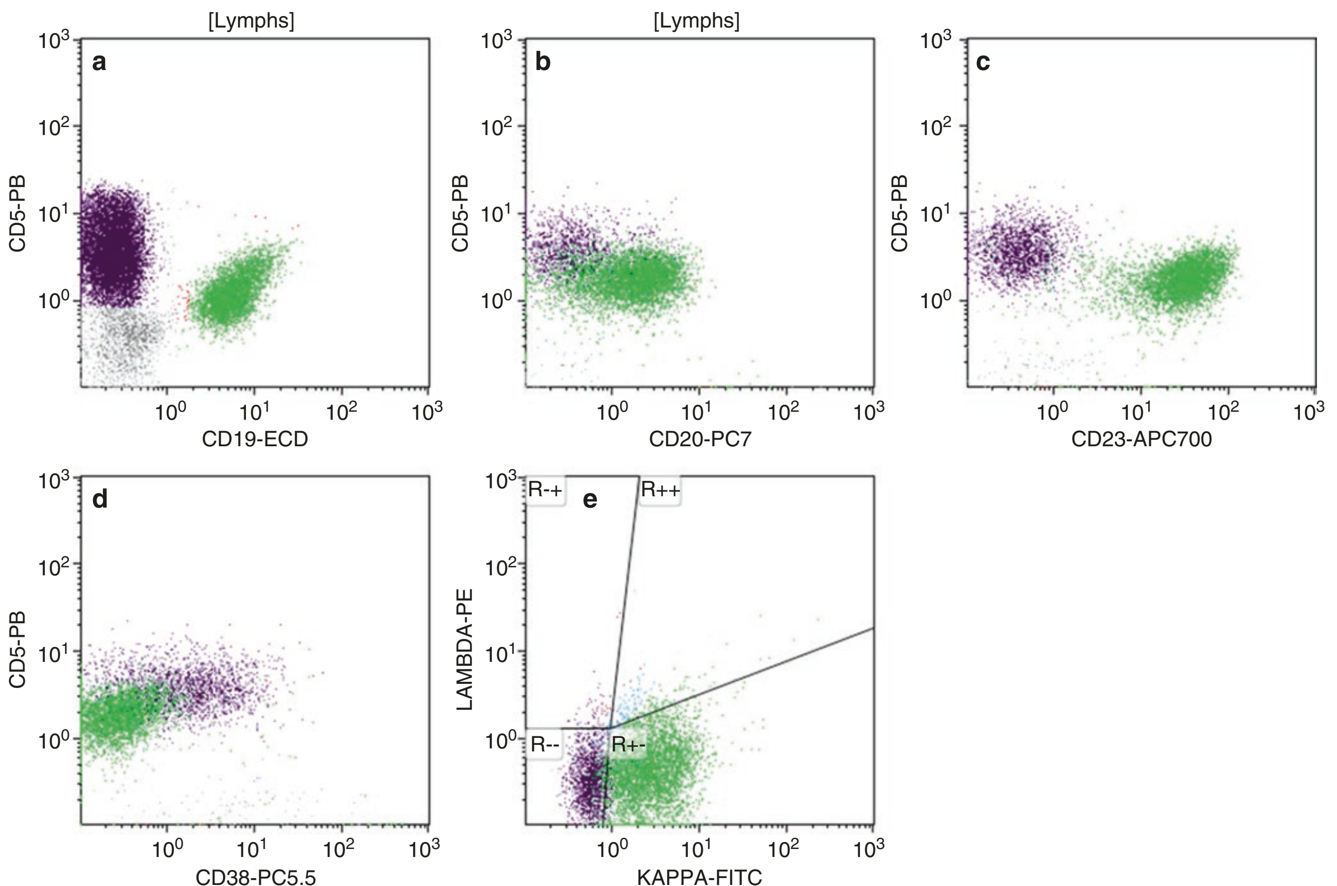

Fig. 7.3 Flow cytometry of CLL: (**a**) The CLL cells are marked *green* and express CD19 (**b**) The CD5 + CLL cells express dim-negative CD20 with slightly dimmer CD5 compared to the T-cells (*violet*). (**c**) There is co-expression of CD23, but the CLL cells are negative for CD38 (**d**) and express dim surface-restricted kappa immunoglobulin light chain (expression level is barely more than the background light chain-negative T-cells) (**e**)

lost after the germinal center reaction in the process of memory B-cell formation. Some have suggested that CD23 correlates with proliferative activity in CLL [83]. Most cases of CLL show expression of CD23 (Fig. 7.3), in contrast to other B-cell neoplasms, such as mantle cell lymphoma. FMC7, an epitope on the CD20 molecule that is expressed by most MCL, is often negative in CLL, although up to 15% of cases can be positive for this marker [84, 85], especially in cases with bright CD20 and Ig positivity.

Bcl-2 is overexpressed in CLL and is related to resistance of apoptosis in the tumor cells, conferring a survival advantage [86]. The mechanism of overexpression in follicular lymphoma is related to the recurrent translocation t(14;18). This is not the case with CLL; rather, hypomethylation of DNA is felt to be the mechanism driving Bcl-2 overexpression [87]. With the advent of selective inhibitors of Bcl-2, the overexpression of the molecule on CLL cells is now more clinically relevant [88, 89].

CD11c, a marker often seen in hairy cell leukemia and B-cell prolymphocytic leukemia, can be observed in CLL. However, the intensity of positivity in cases of CLL, when evaluated by flow cytometry, is usually less than what is observed in hairy cell leukemia (dim negative in most cases), providing a clue to differentiate the two neoplasms using this marker [90].

Among CLL prognostic markers, CD38 and ZAP-70 are the most studied and prognostically important. CD38 is a membrane protein that functions as an adenosine diphosphate ribosyl cyclase. Downstream, this enzymatically controls intracellular calcium levels and is a marker for cell activation [91]. Analysis by flow cytometry would yield three patterns of CD38 positivity: uniformly negative (Fig. 7.3), uniformly positive, and a bimodal pattern with only a subset of the CLL population showing expression [92]. The latter two patterns are associated with unmutated *IGHV* in most cases, which is implicated in poor prognosis. Although a cutoff of 20% positive cells in the tumor has been proposed by some authors, the presence of smaller CD38+ subpopulations (e.g., <10%) may be significant to predict unmutated *IGHV* status. A subset of cases, up to 30% in some studies, shows discordant results with *IGHV* mutation status. In this regard, CD38 positivity is thought to be an independent prognostic factor in CLL [93, 94].

ZAP-70 is not normally expressed in B-cells, but is a tyrosine kinase involved in T-cell receptor signal transduction [95]. The expression of ZAP-70 facilitates the recruitment of Syk to the BCR complex resulting in additional increased BCR signaling. Compared to CD38 expression, ZAP-70 shows more concordance with unmutated *IGHV* status, with up to 97% of ZAP-70-positive cases showing unmutated *IGHV* [95, 96]. Protein expression can be tested by either flow cytometry or immunohistochemistry. In flow cytometry analysis, ZAP-70 in CLL is considered positive when over 20% of tumor cells show expression [97]. In conjunction with CD38 and mutational analysis, three prognostic subgroups can be determined: those with concordant results (ZAP-70+, CD38+, unmutated *IGHV*) have the worst prognosis, while patients without ZAP-70 or CD38 expression and mutated *IGHV* have better prognosis. Those with discordant results have a prognosis intermediate between the two [98, 99]. It is now known that CD38, CD44, CD49d, and matrix metalloproteinase-9 (MMP-9) expressed on ZAP-70+ CLL cells form a macromolecular complex which promotes cross talk between BCR signaling and CD44 further affecting CLL cell migration and homing [100].

A small subset of CLL cases can exhibit variant immunophenotypes and are sometimes termed "atypical" CLL. Some cases can show bright CD20 or sIg expression, and have been correlated with the presence of trisomy 12 [101, 102]. As mentioned above, about 15% of cases can be positive for FMC7. Lack of CD5 co-expression has also been reported, and thought to confer a worse prognosis than CD5+ cases [103].

On the other hand, the classic immunophenotype of CLL can be seen in other lymphomas, including MCL and MZL [104, 105]. Thus, the typical findings on flow cytometry and/or immunohistochemistry are not entirely sensitive nor specific. Indeed CD5 positivity has been described in all other non-CLL small B-cell lymphomas [106]. This highlights the importance of morphologic correlation when evaluating all cases of B-cell lymphoproliferations.

Some of the more recent immunohistochemical markers of CLL include CD200 and lymphoid enhancer binding factor 1 (LEF1). CD200 is a glycoprotein belonging to the immunoglobulin superfamily. It is expressed in most cases of CLL in contrast to MCL, giving it diagnostic utility in cases where the immunophenotyping results may be overlapping between the two entities [107]. Some have suggested a prognostic utility as well, with cases showing strong expression of CD200 having a better overall survival and longer time to treatment [108]. LEF1, on the other hand, is a transcription factor related to the Wnt pathway that helps to regulate genes associated with cell death and survival [109]. LEF1 expression was also found in CD19+/CD5+ B-cells in patients with monoclonal B-cell lymphocytosis, suggesting that this pathway may play an early role in the leukemogenesis of CLL. Most cases of CLL show expression of LEF1 in contrast to MCL [110, 111]. Recent studies also show that strong expression of LEF1 is an adverse prognostic factor [112].

In addition to these diagnostic and prognostic markers, CD2 and CD13 have both been aberrantly described to be expressed in cases of familial CLL [113]. Given the immune derangements consequent to CLL, expansions of certain unusual T-cell populations have been noted in CLL including CD4 + CD8+ populations in line with evidence indicating expansions of these population in elderly patients and CLL-like MBL [114, 115].

Transformation in CLL

The development of an aggressive lymphoma either synchronously or subsequent to a diagnosis of chronic lymphocytic leukemia (CLL) is called "Richter transformation" (RT) and is the accepted definition in the 2008 (and 2016 revision) WHO classification of hematopoietic tumors [11, 17, 116]. Two distinct pathologic variants are described, namely diffuse large B-cell lymphoma (DLBCL) variant (90% of all RT) and the less common Hodgkin lymphoma (HL) variant (10% of all RT) [117]. The former occurs ~2 years after a CLL diagnosis (including in treated and untreated patients) while the latter occurs at a median of 6 years subsequent to a diagnosis of CLL mostly in treated patients [118, 119].

Diffuse Large B-Cell Lymphoma-Richter Transformation (DLBCL-RT)

General Considerations

CLL patients with significant lymphadenopathy, certain genetic polymorphism, and somatic *NOTCH1* carry an increased risk of transformation [7, 120–123]. While the diagnosis of DLBCL-RT is fairly straightforward in most instances, there are three situations in which a diagnosis of DLBCL-RT should not be rendered: (1) Cases of CLL with numerous expansive and at times confluent growth centers: While such "accelerated" cases exhibit more aggressive clinical course with a median overall survival intermediate between typical CLL and DLBCL-RT, the current upcoming revisions note that these cases should not be termed as DLBCL-RT [124]. (2) The occurrence of EBV+ aggressive lymphomas after T-cell-depleting therapies for CLL including alemtuzumab, and as such these malignancies are considered to be secondary to iatrogenic immunosuppression and do not warrant a diagnosis of DLBCL-RT [125]. However, clonally identical B-cells have been reported to be detected in the both the CLL component prior to therapy and the post-alemtuzumab B-cell lymphoproliferation [126]. (3) Lastly, identification of small CD5+ B-cell clones in staging marrows of patients with extramedullary DLBCL without prior/concurrent history of CLL is likely unrelated biologically to the DLBCL and such cases are best considered as de novo DLBCLs.

Histopathology

DLBCL-RT occurs typically as a nodal disease but may also involve extranodal tissues including peripheral blood and bone marrow (Fig. 7.4). Involved lymph nodes show partial or total effacement of the nodal architecture by a DLBCL component with variable amounts of associated CLL component, which is spatially separate or intimately admixed with the DLBCL component. Cytologically, the DLBCL exhibits immunoblastic cytomorphology (with prominent eosinophilic nucleoli and moderate amount of cytoplasm, Fig. 7.4) but cases with centroblastic morphology and in rare instances plasmablastic/plasmacytic morphology has also been described [127–129]. A small proportion of cases may be positive for EBV with coexistent herpes simplex infection, which may be apparent as areas of extensive necrosis with typical Cowdry Type A inclusions. Prior fludarabine exposure increases the risk of HSV infection. Immunophenotypically, the DLBCL-RT exhibits non-GCB phenotype (CD10-, BC6-, MUM1/IRF4+) phenotype in 62–100% of cases based on the Hans classifier with variable expression of CD5 (32–77%) and CD23 (14–80%) [7, 130] (Fig. 7.4). Expression of Mum-1/IRF4 is in keeping with the phenotype of activated B-cell (ABC)/IRF-4- DLBCLs which have worse outcome compared to cases of de novo DLBCLs (Fig. 7.4). Specifically among DLBCL-RTs, there is overrepresentation of non-germinal center B-cell phenotype compared to de novo DLBCLs where the frequency is much lower [131–133].

Given the prognostic relevance of ZAP-70 and p53 in CLL, there has been interest in relevance of these two proteins in the DLBCL-RT. One recent study including 34 DLBCL-RTs noted that ZAP-70 expression by immunohistochemistry in DLBCL-RT (Fig. 7.4) was less frequent (13% of all DLBCLs tested) compared to the CLL component (66%) [130]. However, ZAP-70 status did not have any prognostic value. However, a significant proportion of DLBCL-RT in this study expressed p53 [130]. This observation is in congruence with reported genomic studies corroborating overrepresentation of *TP53* mutations in CLL and their corresponding DLBCL-RT tissues compared to CLLs that have not transformed to DLBCL with reported *TP53* disruption and *CDKN2A* as a main mechanism of transformation in CLL [134]. This study further identified a second major subgroup characterized by the presence of trisomy 12 comprising a third of the cases in the series indicating the existence of distinct pathways of transformation to DLBCL in CLL.

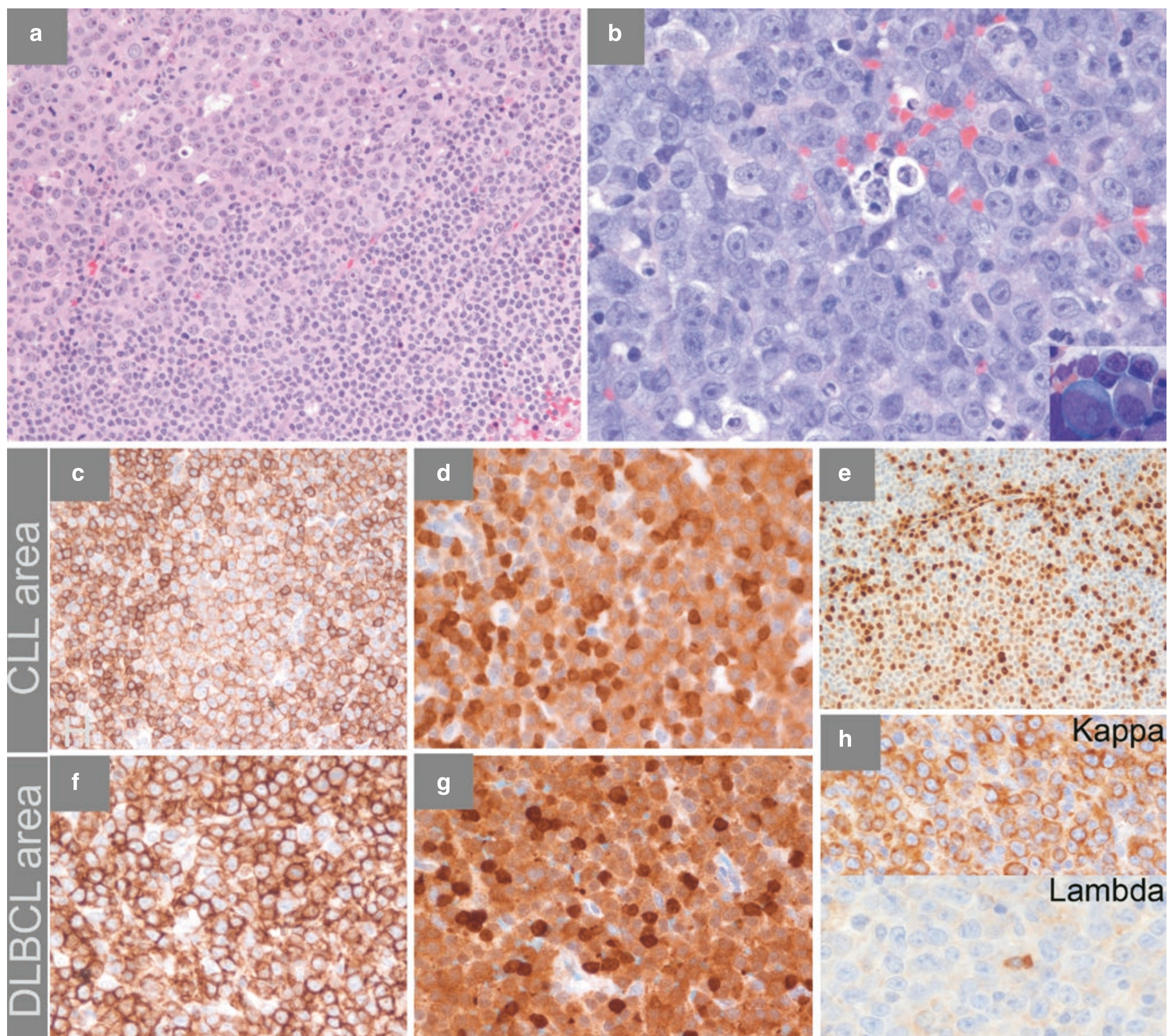

Fig. 7.4 Diffuse large B-cell lymphoma-Richter transformation (**a**). Bone marrow with spatially distinct large cell (*top left*) and CLL component (*bottom right*). (**b**) Large cell area at high power with prominent immunoblastic cytomorphology (inset shows large cells on marrow aspirate smears). (**c**, **f**) demonstrate CD5 immunostain in the CLL (proliferation centers) and DLBCL areas. Both components in this case are CD5 positive and ZAP-70 positive (**d**, **g**) In both stains, background T-cells express bright CD5 and ZAP-70. (**e**) Mum-1/IRF4+ staining within growth centers (*lower half of figure*) with an associated plasmacytic component at the periphery (*upper half of figure*). (**h**) The large-cell component shows restricted kappa light chain by immunohistochemistry (*top panel*)

Clonality Studies and Differential Diagnostic Considerations

Despite the frequently differing immunophenotype of DLBCL-RT in comparison with the prior CLL, clonality studies of IG genes and *IGHV* segment usage indicate that both components are frequently clonally related in up to 80% of cases [123, 130]. Also, with respect to *IGHV* status and cell of origin status, de novo DLBCLs differ from DLBCL-RT in frequently carrying mutated *IGHV* genes as opposed to DLBCL-RT which harbor unmutated *IGHV* genes. Another important differential in this setting is de novo CD5+ DLBCL since CD5 expression is otherwise unusual for de novo DLBCLs. However, CD5+ DLBCLs, despite being clinically/immunophenotypically similar (advanced age and stage at presentation, non-GC phenotype) to DLBCL-RT, in contrast carry mutated *IGHV* (up to 80% of cases) and are frequently CD23 negative as opposed to CD5+ transformation of B-CLL which is unmutated in most instances [135–137].

Clonally unrelated DLBCL-RT were noted to harbor less frequent *TP53* mutations, less frequent B-cell receptor stereotypy,

and longer outcome compared to clonally related cases, nearly 47% of which harbored *TP53* aberrations [123]. This study noted MYC aberrations in up to 26% of DLBCL-RT cases. It is unclear as yet if MYC IHC positivity/aberrations in DLBCL-RT are independently prognostic or are only reflective of an aggressive *TP53* mutation-driven biology. There is some evidence that it is implicated in transformation [123, 138, 139]. One recent study noted that *MYC* aberrations are frequently acquired during the course of the disease and when present as part of a complex karyotype were associated with the development of RT and adverse outcome in contrast to cases harboring *MYC* aberrations as a part of a non-complex karyotype which responded well to standard risk-adapted therapy [140].

Classical Hodgkin Lymphoma-Richter Transformation (CHL-RT)

This variant of CLL transformation is 10–20 times less common than DLBCL-RT and occurs in less than 1% of CLL patients [118, 141–143]. In one of the first studies from Mayo Clinic looking at the incidence of CHL-RT in CLL patients within a prospective and a nested cohort (including only the subset with newly diagnosed CLL within the study period 1995–2011), the 10-year risk of CHL was 0.5% [118]. Compared to de novo CHL patients, CHL-RT patients in this study exhibited shorter overall survival and frequently presented at higher IPS stage and furthermore patients receiving any CLL therapy prior to the CHL transformation did worse compared to those who did not. Although 67% of cases in this series developed EBV+ CHL-RT, it was not possible to draw conclusions on outcome stratified by EBV status due to confounding prior nucleoside analog therapy in most patients within the EBV+ subset.

Two different histologic patterns of CHL have been described in the context of CHL-RT [59, 144, 145]. “Type I” pattern referred to the occurrence of isolated Hodgkin/Reed-Sternberg (HRS) cells scattered in a background of CLL lacking the typical polymorphous cellular milieu of CHL (viz. fibrosis, eosinophils, and neutrophils). “Type II” denoted cases with HRS cells scattered within typical CHL microenvironmental milieu (Fig. 7.5). These lesions may demonstrate synchronous composite histology with CLL and

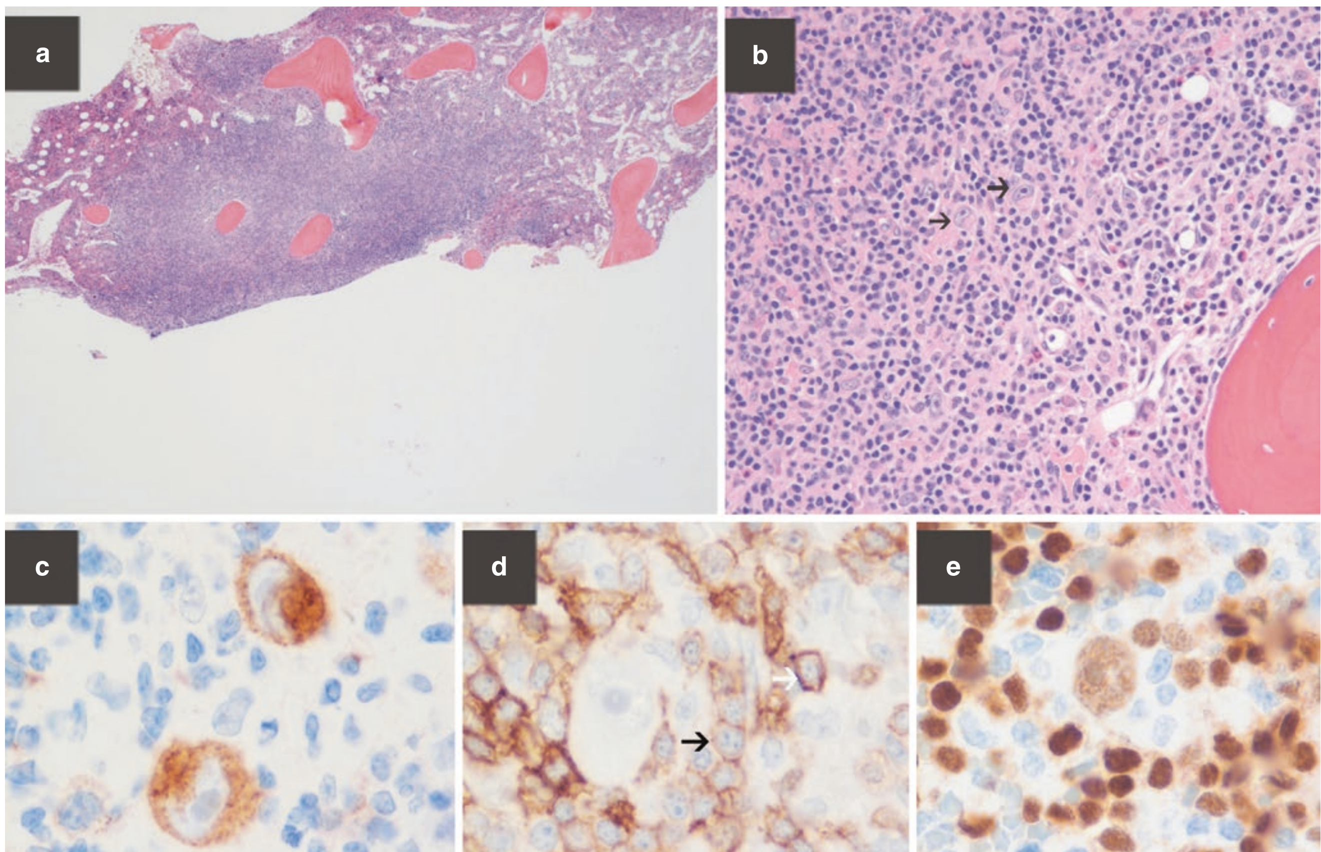

Fig. 7.5 Hodgkin lymphoma-Richter transformation in a CLL patient with two prior treatments including fludarabine, cyclophosphamide, and ofatumumab as well as acalabrutinib (irreversible Btk inhibitor) with progressive lymphadenopathy in whom bone marrow biopsy was performed for cytopenias. (**a**) Low-power view of marrow core biopsy showing nodular interstitial lymphohistiocytic aggregates which at high power (**b**) shows scattered HRS cells (*arrows*) amidst a polymorphous cellular milieu comprising lymphocytes, histiocytes, and few eosinophils. (**c**) The HRS cells express CD30 and are rimmed by CD5+ T-cells. (**d**) The CD5 immunostain shows T-cells with bright CD5 (*white arrow*) and CD5-dim lymphoid cells corresponding to CLL cells (*black arrow*) better highlighted on Pax5 immunostain (**e**)

Hodgkin components (either in the same node or different sites) or as metachronous development of CHL subsequent to CLL diagnosis. Progression from type I to type II histology may occur in some instances.

Immunophenotypically, the HRS cells exhibit a similar phenotype as seen in de novo classical Hodgkin lymphoma (CD30+, CD15+/variable, CD45-, weak Pax-5) (Fig. 7.5) with variable downregulation of other B-cell markers (CD20, CD79a, Oct-2, Bob.1) [117]. Most cases are usually positive for EBV (71%) in the HRS cells by in situ hybridization or EBV-LMP1 immunohistochemistry [141, 146]. While earlier biologic studies informed us regarding the occurrence of some clonally related and other clonally unrelated cases with inverse association of EBV and clonal relatedness, these were mostly single reports and small case series where results were not generalizable [59, 144, 145, 147]. In one of the largest series of such cases published recently including 26 type I Hodgkin-like lesions, the authors examined the EBV status, clonal relatedness (of both CLL and CHL components), and ZAP-70 status [146]. In this study, the authors noted that the CHL component was clonally related in about 40% of cases and interestingly all CLL negative for ZAP-70 were clonally related to the paired CHL component. This is in contrast to DLBCL-RT wherein all or most cases of DLBCL-RT are clonally related to the CLL component regardless of *IGHV* status. Only age > 70 years was predictive of adverse outcome in this study. While it was controversial from earlier studies whether type I lesions should be considered transformation, the study by Chen noted that cases with type I histology had an overall survival similar to cases with type II histology.

Minimal Residual Disease Testing in CLL by Flow Cytometry

With the progress made with combinations of chemotherapy and immunotherapy in CLL, there is growing recognition of association of MRD-negative status and other outcome measures. Several studies have demonstrated the utility of MRD-based flow cytometric testing in a variety of clinical settings including first-line, salvage/high-risk, and post-allogeneic transplant settings and it is thought that it may perhaps be useful even as a clinical trial endpoint with novel immunotherapy agents [148].

While earlier studies examined MRD status using two-, three-, or four-color tubes including CD19, CD5, and Kappa/lambda [149–152], the first major approach to standardized combinations was proposed by the European Research initiative in CLL (ERIC) which examined 728 paired blood and marrow samples and demonstrated that there was a high concordance between blood and marrow although marrow FC was required for definitive assessment of MRD-negative status 3 months after alemtuzumab therapy. There was concordance of the FC-based MRD status and real-time quantitative allele-specific oligonucleotide (RQ-ASO) immunoglobulin heavy-chain gene (IgH) polymerase chain reaction (PCR) [153]. This study identified specific useful four-color FC combinations including CD19/CD5 with CD20/CD38, CD81/CD22, and CD79b/CD43 as the most useful combination for detecting MRD at the 0.01% level. These observations were then extended in a follow-up study demonstrating equivalence of a harmonized six-color panel with the previously proposed four-color panel in detecting residual CLL at the 0.01% level utilizing the same marker combinations including CD20, CD22, CD38, CD43, CD79b, and CD81 [154]. This latter study utilized a threshold of at least 50 events to define a CLL population with the need for CD3 for determining the limit of detection. Very recently, a combination of markers proposed by ERIC were tested in an eight-color panel with the ability to detect MRD at 0.007% level [155].

Differential Diagnosis

In the assessment of a patient with B-cell lymphocytosis, the differential diagnosis can be broad and range from benign, reactive conditions to a number of B-cell lymphomas. An important and useful first step in differentiating between these conditions is a systematic assessment of lymphocyte morphology. Generally, uniformly small-to-medium cells in the peripheral blood is suggestive of CLL, while variability in cell size and shape is more indicative of a reactive condition or another lymphoma. Clinical history is also important, as lymphocytosis in a young patient is more likely to represent an infectious process, such as a viral illness, rather than lymphoma. Polyclonal B-cell lymphocytosis, a rare condition more common in young female smokers and associated with HLA DR7 [156], may also enter the differential. It is characterized by a persistent B-cell lymphocytosis of mostly small, round lymphocytes with only mild morphologic variability, closely resembling CLL. Often, flow cytometry and/or clonality studies may be necessary to exclude lymphoma.

Distinction between CLL and other mature B-cell neoplasms can be more difficult especially when the morphology of CLL is atypical. In the early 1990s, a flow cytometric scoring system was proposed by Matutes and coworkers which examined the expression levels of CD5, CD22, CD23, FMC7, and surface immunoglobulin. CLL with a typical expression pattern (CD5+, CD23+, FMC7-, dim sIg, and dim CD22) were assigned a score of 5 [157]. However, in these cases, particular attention to morphologic features, along with correlation with immunophenotype, can aid in arriving at the correct diagnosis.

Mantle cell lymphoma, particularly the small-cell variant, is possibly the most important differential diagnosis, due to some similar morphologic and immunophenotypic features with CLL, despite its significantly poorer prognosis. Leukemic presentation can occur in MCL [158] and the cells can resemble CLL, namely medium-sized cells with scant cytoplasm. However, the nuclei in MCL are often cleaved and the chromatin usually is more dispersed. In tissue sections, proliferation centers that are characteristic of CLL are notably absent in MCL. Additionally, prolymphocytes are not found in cases of MCL. Although both lymphomas share expression of CD5, in contrast with CLL, MCL shows strong expression of CD20 and CD79b. A key differentiating factor is the expression of cyclin D1 in MCL, which is correlated with the presence of t(11;14)(q13;q32). In cases where cyclin D1 is absent, SOX11 is present in most cases [159]. Absence of SOX11 was found to confer better survival and a more indolent form of MCL [160].

Uncommonly, CLL can exhibit plasmacytoid features, morphologically resembling lymphoplasmacytic lymphoma (LPL). The latter contains a mixture of plasma cells, plasmacytoid cells, and small lymphocytes. The peripheral blood may also exhibit red cell agglutination, indicative of the presence of antibodies on the red cell surface (Fig. 7.6). The bone marrow may show diffuse infiltration (Fig. 7.6). Mast cells are typically increased in LPL. Proliferation centers are absent and CD5 is generally not expressed although CD10 can rarely be expressed in LPL. Expression of CD20, surface immunoglobulin, and CD79a is usually higher than in CLL. Testing for serum IgM paraprotein and presence of concurrent *MYD88* L265P mutation (which is now known to be present in almost all LPL) allows confirmation of LPL [161].

CLL with increased prolymphocytes or in prolymphocytic transformation can resemble B-cell prolymphocytic lymphoma (B-PLL). However, as defined by the WHO, the diagnosis of B-PLL requires that the neoplastic cells comprise 55% or more of the peripheral white blood cells (Fig. 7.6). Although lymph node involvement is uncommon in B-PLL, if present, proliferation centers are not observed. CD20 expression in B-PLL is typically bright, in contrast to most cases of CLL.

The distinction between follicular lymphoma and CLL is often straightforward. Although at times the pseudofollicles of CLL can resemble the true neoplastic follicles of follicular lymphoma and the neoplastic cells can appear slightly cleaved, attention to all architectural and morphologic features, along with immunophenotypic profiles, would aid in the diagnosis. When evaluating bone marrow samples, follicular lymphoma typically occupies the paratrabecular spaces, rather than interstitial or diffuse involvement that is characteristic of CLL.

Splenic marginal zone lymphoma presents with peripheral blood and spleen involvement without significant lymphadenopathy. The neoplastic cells characteristically have polar villi (Fig. 7.6) with a chromatin pattern quite similar to CLL cells. The bone marrow shows intra-sinusoidal infiltrates (Fig. 7.6), which are very unusual in CLL. Immunophenotypically, there is strong expression of FMC7 and B-cell markers, in contrast to CLL cells. CD5 can be expressed in some cases of splenic marginal zone lymphomas, and some suggest that cases with this immunophenotype present with higher levels of lymphocytosis. However, there was no difference in overall outcome in CD5+ lymphomas as compared to CD5-negative cases [162].

Differentiation of hairy cell leukemia (HCL) from CLL is usually not very challenging. HCL often presents in the peripheral blood, spleen, and bone marrow, without significant peripheral lymph node involvement. The classic cytomorphology of HCL cells is medium-sized lymphoid cells with circumferential hairlike cytoplasmic projections (Fig. 7.6). The nuclear membrane is often oval or indented and the chromatin is slightly more dispersed than the clumpy chromatin pattern seen in CLL cells. Examination of the bone marrow often shows diffuse involvement and a “fried-egg” appearance of the neoplastic cells (Fig. 7.6). Immunophenotypically, HCL cells have strong expression of sIg and B-cell antigens, such as CD20. More specific hairy cell markers such as CD103, CD25, and CD123 are often present. Immunohistochemical stains for annexin A1, tartrate-resistant acid phosphatase (TRAP) (Fig. 7.6), and DBA.44 on tissue sections can also be helpful. CD5 is often negative.

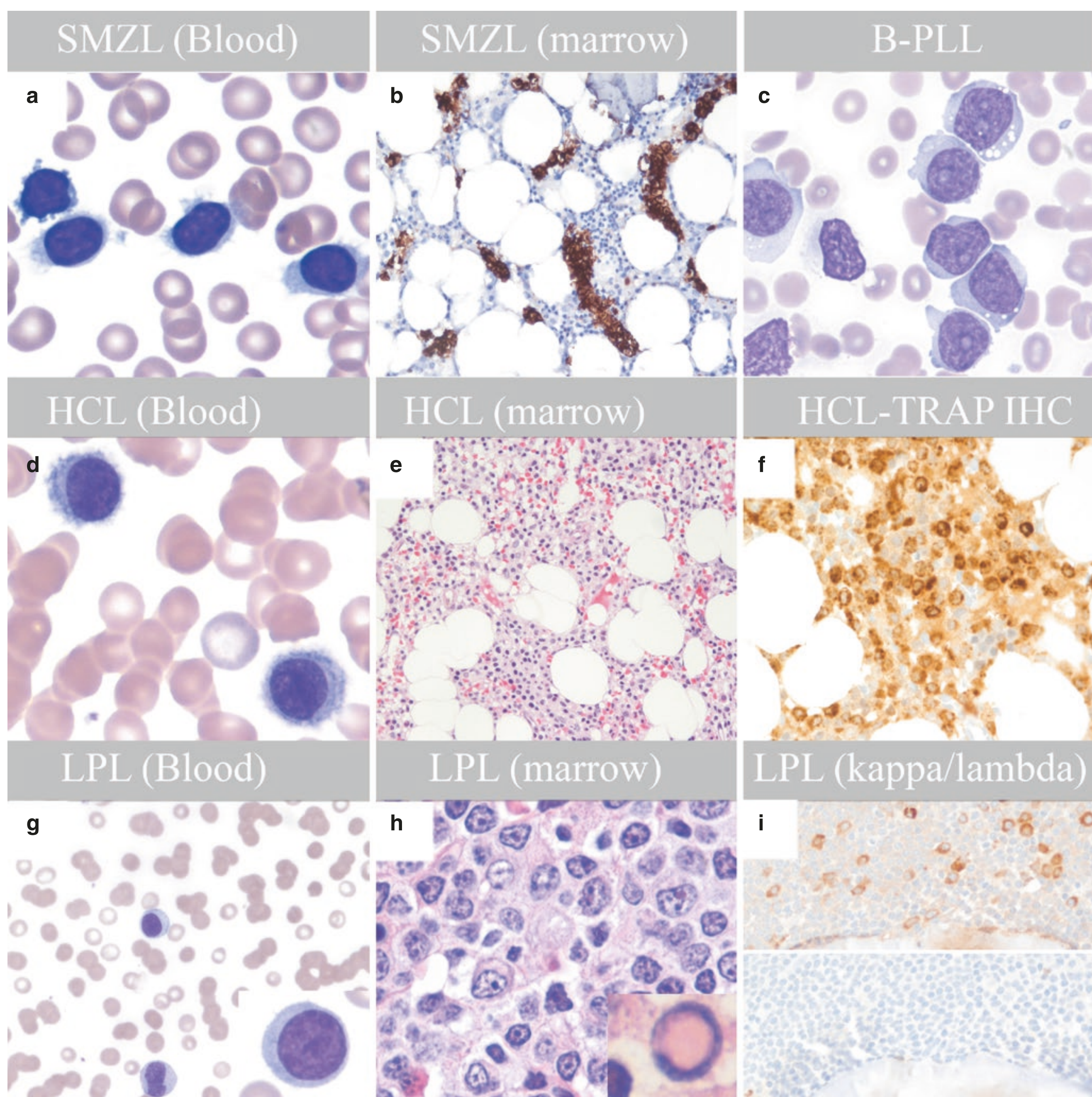

Fig. 7.6 Differential diagnostic considerations. (**a**) Splenic marginal zone lymphoma (SMZL) involving peripheral blood with polar villous projections (**b**) SMZL in marrow characteristically shows intra-sinusoidal infiltration pattern of lymphoma cells more frequently which is a useful diagnostic feature (CD20 immunoperoxidase stain). There is often heterogeneity of cell size in such cases but typical clumped nuclear chromatin and smudge cells as seen in CLL are not present. (**c**) B-Prolymphocytic leukemia (B-PLL) containing uniform population of large cells with distinct nucleoli and moderate amount of cytoplasm. With growing understanding, very few of such cases represent CLL in prolymphocytic transformation. (**d**) Hairy cell leukemia (HCL) involving peripheral blood with typical hairy cytomorphology. (**e**) HCL involving bone marrow core biopsy with typical fried-egg appearance (**f**) Tartrate-resistant acid phosphatase (TRAP) immunohistochemistry strongly positive in HCL. (**g**) Lymphoplasmacytic lymphoma involving peripheral blood with marked red cell agglutination (*inset:* typical cell with morphology intermediate between lymphocytes and plasma cells). (**h**) Core biopsy of LPL showing extensive infiltration by LPL cells (*inset:* Dutcher body, defined as intranuclear inclusions of cytoplasmic immunoglobulin) (**i**) Kappa light-chain restriction with LPL cells demonstrated via IHC on core biopsy. Although the morphologic distinction is easy in most instances, HCL, SMZL, and B-PLL may variably express CD5 and CD23 in a significant proportion of cases and may pose diagnostic confusion from a flow cytometric perspective

Conclusion

As our understanding of the pathogenesis of CLL grows, "bench-side" discoveries are increasingly finding their way to the "bedside," with more examples of targeted therapy and precise prognostication discovered each year. The molecular genetic profile of each tumor can aid clinicians in providing the appropriate therapy, a delicate balance between effective medicine against the tumor and minimizing any toxic side effects. Despite these advances, the diagnosis of CLL is still dependent on analysis using the classic, ageless light microscope and evaluation of characteristic morphologic and architectural features. With the additional help of flow cytometry and immunohistochemistry, CLL can be differentiated from its mimics and a precise diagnosis can be rendered by the pathologist. Only after this point do molecular tests have meaning and a context can be drawn that links the basic science discoveries in CLL to the practical treatment and care of the patients we serve.

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8 The Genomic and Epigenomic Landscape of Chronic Lymphocytic Leukemia

Jonathan C. Strefford, Renata Walewska, and David G. Oscier

Introduction

Since the previous edition of this book, our understanding of the cytogenetics and genomics of chronic lymphocytic leukemia has taken a quantum leap forward, principally the result of seminal technological advances (Fig. 8.1). In the 1970s, the development of polyclonal B-cell mitogens culminated with the discovery of the first cytogenetic abnormality in CLL, trisomy 12, in 1980, shortly followed by the interstitial deletion of 13q14 [1, 2]. With the advent of more effective mitogens, most recently CpG oligonucleotides combined with interleukin 2 [3, 4], the majority of cases of CLL yield metaphase preparation and clonal abnormalities are identifiable in approximately 80% [5]. In contrast to many other mature B-cell tumors, recurrent reciprocal translocations are uncommon in CLL, whereas copy number changes, particularly deletion events such as those affecting 11q, 13q, and 17p, and trisomy of chromosome 12 occur frequently.

In the 1980s, fluorescent *in situ* hybridization (FISH) approaches overcame the need for dividing cells, thereby permitting the analysis of interphase nuclei. The seminal study by Döhner and colleagues established the relative prognostic significance of a panel of recurrent copy number changes, based on the presence of 17p, 11q, and 13q deletions, and trisomy 12, with 13q deletions (as a sole abnormality) and 17p deletion being the markers of best and worst prognosis, respectively [6]. Similar data can now be obtained using either multiplex ligation-dependent probe amplification (MLPA) or quantitative PCR [7–10], although these approaches are rarely used in the clinical setting.

The development of comparative genomic hybridization (CGH) initially with a chromosome template [11], but later with an array-based template [12], allowed the entire genome to be screened for copy number alterations (CNAs) in a single experiment. Currently, copy number changes and loss of heterozygosity (LOH) events can be detected using arrays with more than two million unique genomic features, enabling the identification of genomic alterations of 10–100Kb in size. These studies have identified novel recurrent regions of copy number changes and permitted the gene content of more established lesions to be accurately delineated.

While early molecular studies, employing traditional approaches, identified important mutated cancer genes in CLL, such as *TP53* and *ATM* genes occurring in approximately 80% of patients with *TP53* loss and 40% of patients with *ATM* loss, respectively [13, 14], it was the development of high-throughput massively parallel sequencing that permitted the analysis of the entire CLL genome [15]. Whole-genome (WGS) and -exome (WES) sequencing of more than 1000 CLL patients has led to the discovery of novel mutated cancer genes, positioned within key biological pathways, and has allowed the aforementioned "Döhner" prognostic model to be nuanced with both gene mutation and immunogenetic data [16]. Furthermore, these experiments can be performed with much greater resolution, allowing clinically relevant low-level subclonal mutations to be identified and providing insights into the nature and extent of intraclonal heterogeneity [17, 18]. Indeed, single cells can be isolated using modern flow cytometry or microfluidic approaches and analyzed using next-generation sequencing (NGS) techniques, providing even greater resolution of the clonal architecture of human neoplasms. Methodological advances are also contributing to a greater understanding of the methylome

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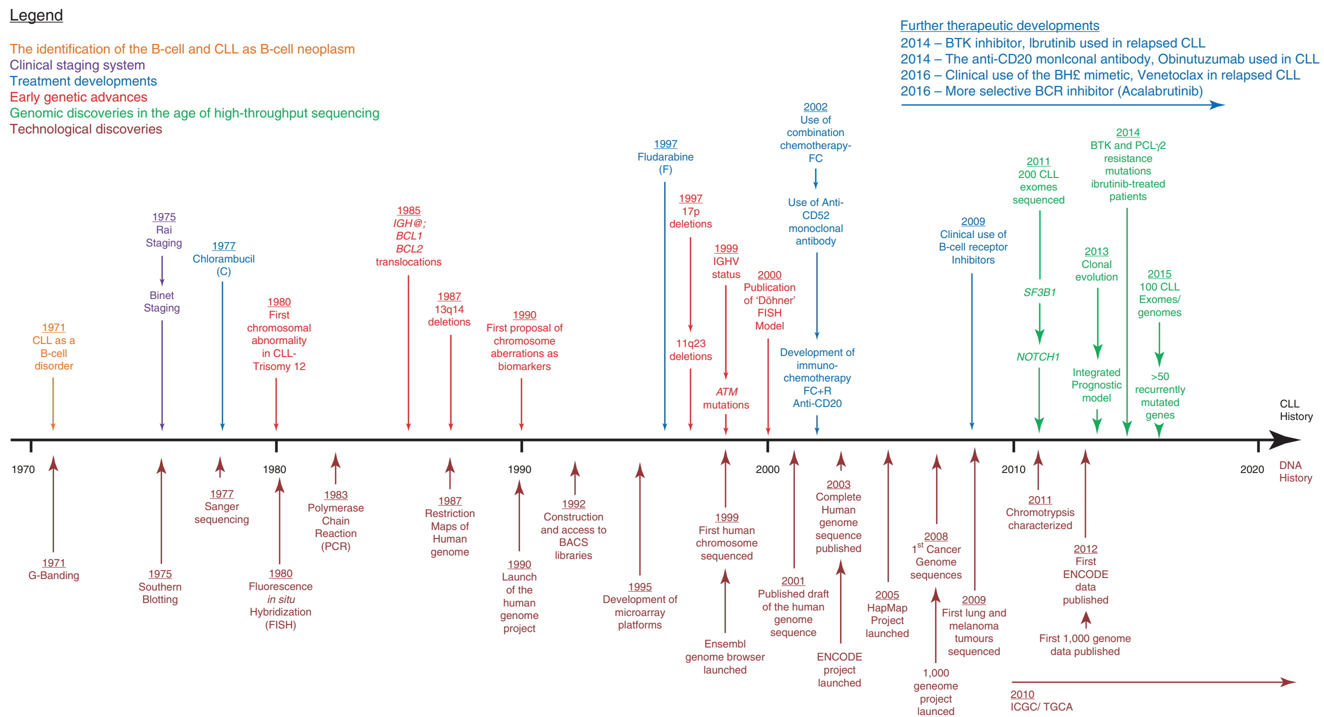

Fig. 8.1 Historical overview of the developments in the field of genomics, and the advancing understanding of the molecular pathogenesis and treatment of CLL

and chromatin landscape in CLL and their relationship to the putative cells of origin of *IGHV*-mutated and unmutated CLL [19–22]. While a complete annotation of the CLL genome and epigenome is in sight, ongoing analysis of the CLL proteome, transcriptome including microRNA expression, and functional consequences of specific genomic and epigenetic abnormalities are beginning to unravel the drivers for clonal selection while also providing prognostic data and identifying therapeutic targets.

In the remainder of this chapter we provide an overview of the genomic landscape, and discuss the commonest genomic abnormalities found in both CLL (Tables 8.1 and 8.2) and Richter's transformation, epigenetic abnormalities, genetic predisposition to CLL, and finally the clinical importance of genomic abnormalities in the management of patients with CLL.

Table 8.1 Recurrent copy number changes in CLL

Gene name	Prevalence (%)	Principal candidate genes	Other candidate genes	References
del(13q)	60–80	*miR-15a/16-1, DLEU2*	*RB1, DLEU7*	[2, 23, 24]
del(11q)	10–20	*ATM*	*BIRC3, MRE11, H2AFX*	[25]
del(17p)	5–50	*TP53*	–	[6]
Trisomy 12	10–15	Unknown	–	[26]
del(6q)	5	Unknown	–	[27]
dup(2p)	5–28	*REL, BCL11A, XPO1*	*MYCN*	[28, 29]
dup(8q)	5	*CMYC*	–	[30]
del(15q)	4	*MGA*	–	[30, 31]
del(3p)	3	*SETD2*	–	[32]

Chromosomal Abnormalities

Deletion of Chromosomal Band, 13q14

Deletion of 13q14.3 is the commonest cytogenetic abnormality in CLL, seen in 50–60% of patients (Table 8.1). Recent WGS data demonstrates the result of interstitial deletions in approximately 60% of patients and translocations involving numerous, sometimes recurring, partner chromosomes (e.g., chromosomes 1, 2, and 14) in the remainder [48]. Approximately 10% of 13q deletions are the result of a complex chain of genomic lesions targeting several chromosomes, characteristic of chromoplexy [49]. 13q14 deletions may be heterozygous (in 70% of cases) or homozygous, where the latter can be the result of copy number-neutral LOH in rare cases [23].

The size of the 13q14 deletion is highly heterogenous in both size and gene content, but a minimally deleted region which includes exons from *DLEU2*, a long noncoding RNA, *DLEU1*, and miRNA 15a/16-1 cluster has been reported [50]. The importance of these genes is demonstrated most

strikingly by a transgenic mouse model, in which either the minimally deleted region encompassing both *DLEU2* and miR 15a/16-1 or miR 15a/16-1 alone was deleted [51]. In both models a clonal B-cell population proliferated in approximately 30% of mice. In the majority of cases the histology and immunophenotype of these B-cell expansions resembled human CD5-positive monoclonal B-cell lymphocytosis (MBL), CLL, or small lymphocytic lymphoma (SLL). A minority of mice developed clonal CD5-negative lymphomas. Bi-allelic deletions and deletions of both *DLEU2* and miR 15a/16-1 were associated with more aggressive disease [51]. In this model, the miRNA cluster regulates the expression of a series of cell cycle genes including *CCND1, CCND2, CCNE1, CDK4*, and *CDK6* which, in turn, regulate transition from G0 to G1 to S phase [51].

Table 8.2 Recurrent mutation genes in CLL, whose importance is evidenced by validation across independent cohorts with associated biological or clinical correlations

Gene name	Gene nomenclature	Approximate frequency (%)	References
Tumor protein p53	*TP53*	5–27	[13]
Ataxia telangiectasia mutated	*ATM*	9–14	[14]
Notch 1	*NOTCH1*	3–24	[33–35]
Splicing factor 3b, subunit 1, 155 kDa	*SF3B1*	5–17	[34, 36]
Nuclear factor of kappa light polypeptide gene enhancer in B-cell inhibitor, epsilon	*NFKBIE*	10	[37]
Paired box 5	*PAX5*	9	[38]-
Ribosomal protein S15	*RPS15*	5–20	[17, 39]
Early growth response 2	*EGR2*	1–8	[18, 37]
Baculoviral IAP repeat containing 3	*BIRC3*	1.5–6	[40]
Chromodomain helicase DNA-binding protein 2	*CHD2*	5	[34]
Mediator complex subunit 12	*MED12*	2–5	[18, 41]
Protection of telomeres 1	*POT1*	5	[34]
Myeloid differentiation primary response gene 88	*MYD88*	3–5	[34, 42]
SET domain containing 2	*SETD2*	4	[32, 38]
F-box and WD repeat domain containing 7, E3 ubiquitin protein ligase	*FBXW7*	4	[36]
SAM domain and HD domain 1	*SAMHD1*	3	[18, 43]
Sucrase-isomaltase (alpha-glucosidase)	*SI*	3	[44]
Exportin 1	*XPO1*	2.5	[42]
V-raf murine sarcoma viral oncogene homolog B1	*BRAF*	2–4	[17, 45]
V-Ki-ras2 Kirsten rat sarcoma viral oncogene homolog	*KRAS*	2	[18, 46]
Interferon regulatory factor 4	*IRF4*	1.5	[17, 38, 47]

Several other studies have shown a correlation between downregulation of miR 15a/16b and upregulation of BCL2 expression [52, 53]. The consequences of loss of DLEU2 and other genes outside the minimally deleted region which nevertheless are deleted in most cases with 13q14 loss are areas of intensive research, where it is clear that 13q deletion size has significant biological and clinical consequences [23, 54, 55].

Genomic Lesions Targeting 11q23

Structural abnormalities of the long arm of chromosome 11 are frequent in CLL, occurring in approximately 20% of patients (Table 8.1). The incidence is higher in advanced- than in early-stage disease and patients frequently have widespread bulky lymphadenopathy. Cytogenetic studies show that most cases with an 11q abnormality have a deletion involving 11q23. Deletions vary in size usually occurring within the 11q21–q25 region. Balanced translocations in this region are rare [54].

The great majority of 11q deletions in CLL result in loss of the ataxia telangiectasia-mutated (*ATM*) gene and approximately 40% of patients with *ATM* loss carry a mutation of the remaining allele (Table 8.2) [55, 56]. Approximately 12% of patients have an *ATM* mutation without an accompanying 11q deletion [57]. In patients with bi-allelic *ATM* abnormalities, the mutation may either precede or follow the deletion of the other allele [40]

As only 40% of 11q deleted cases carry an inactivating mutation of *ATM*, and in vitro studies of the function of the double-stranded DNA repair pathway appear to be preserved in patients with an 11q deletion without an *ATM* mutation, it has been postulated that other genes on 11q may impact disease pathogenesis [40]. The postulated role of other genes on 11q has not been supported by the analysis of genes within the well-defined MDR on 11q, where studies have shown reduced expression of these genes, but have not identified deleterious mutations [57]. However, genomic profiling approaches have shown that 11q deletions are heterogeneous in both size and location, and it has been proposed that other genes involved in DNA damage response, such as Mre11 and *H2AFX*, deleted in 50% and 18% of

cases with an 11q deletion, respectively, might also contribute to leukemogenesis [58].

Another gene on 11q of potential importance is *BIRC3*, a negative regulator of NF-κB signaling. *BIRC3* deletions occur in approx. 80% of cases with 11q loss, where they are always concomitant with *ATM* deletion [56, 57]. Mutation occurs in approx. 2% of cases, where they associate with constitutive noncanonical NF-κB activation in fludarabine-refractory CLL patients with these mutations [40]. However, *ATM* and *BIRC3* lesions often coexist in the same patient, and it is an *ATM* mutation that is most associated with poor outcome in del(11q) CLL [57]. 11q deletions can also include the microRNA (miR) cluster that included miR-34b and miR-34c. These miRs interact with p53, with reduced expression and hypermethylation of miR-34b/c locus occurring preferentially in non-11q deleted cases [58].

Deletion and Mutations of the Tumor-Suppressor Gene, *TP53*

Structural abnormalities of chromosome 17p, including deletions, translocations (usually unbalanced), and isochromosome 17q, are detectable cytogenetically in less than 5% of patients with early-stage CLL rising to over 30% in patients with advanced chemo-refractory disease (Table 8.1). In routine practice it is usual to screen for 17p loss using a FISH probe encompassing the *TP53* gene. Factors that can influence interphase FISH results such as the choice of probe, number of cells counted, scoring of all lymphocytes or just clonal B cells, and choice of cutoff are of particular importance for TP53 screening in view of the clinical significance of *TP53* loss. 80–90% of cases with *TP53* loss have a mutation of the remaining allele (Table 8.2). 3–5% of patients acquire a *TP53* mutation without loss of the other allele [59–62]. Bi-allelic *TP53* mutation also occurs in rare cases, due to either a second unique mutation or the presence of 17p copy number-neutral loss of heterozygosity [59].

Using traditional molecular screening approaches, *TP53* mutations can be identified in approximately 9% of untreated CLL cases, with loss of the second allele seen in the majority of mutated cases (Table 8.2). Differences in the incidence of *TP53* loss and/or mutation among series reflect differences in patient populations and methods for screening for *TP53* mutations. The majority of *TP53* mutations are missense and located within the DNA-binding domain of p53 encoded by exons 5–8. Approximately 20% of mutations occur within 6 “hot spots.” The *TP53* mutation profile is similar in both previously untreated and treated patients suggesting that chemotherapy selects preexisting small p53 mutated clones [59]. The presence of a *TP53* mutation, thought to be an event preceding clonal evolution [60], is a strong independent marker of adverse survival and a powerful predictor of poor response to chemo-immunotherapy, and therefore has direct implications on treatment decisions [61]. Low-level subclonal mutations, beyond the resolution of Sanger sequencing (down to 0.3% of the cancer cells), reside in approximately 9% of untreated CLL, mutations that expand to become more clonal in sequential samples from patients that ultimately relapse [61, 62]. Patients with these subclonal *TP53* mutations show the same clinical phenotype and poor survival [61] as patients with clonal mutations and carry a higher risk of mutation selection by therapy. Identifying *TP53* defects early in their evolution may enable improved clinical management of high-risk CLL. It is important to mention a rare subset of early-stage CLL patients with mutated *IGHV* genes and *TP53* abnormalities do exhibit a more stable disease course [63].

Functional studies in which double-stranded DNA breaks are induced in leukemic cells in vitro and the expression of p53 and its downstream targets such as p21 and miR34a are measured are also able to detect TP53 abnormalities in CLL [63]. More recent studies suggest that primary abnormalities of p21 and miR34a expression in patients with no detectable TP53 abnormality may also cause p53 dysfunction and can be associated with poor clinical outcome [30, 32]. *TP53* abnormalities are frequently associated with complex genomic abnormalities and a poor outcome as discussed below.

Trisomy 12

Trisomy 12 is the most frequent numerical chromosome abnormality in CLL occurring in approximately 10% of patients (Table 8.1). It is usually the primary cytogenetic abnormality detectable at diagnosis; acquisition of trisomy 12 during the course of disease is extremely rare. There is a strong but as-yet unexplained association between trisomy 12 and both atypical lymphocyte morphology and an atypical immunophenotype [64]. The role of trisomy 12 in the pathogenesis of CLL remains unclear. Structural abnormalities of chromosome 12 may result in a partial trisomy 12 with duplication of the region between q13 and q22. This region includes the MDM2 gene which is overexpressed in patients with trisomy 12 [65]. Trisomy 12 may occur as the sole cytogenetic abnormality in CLL but is frequently accompanied by additional trisomies, particularly of chromosomes 19 and 18 [66], deletion of 13q14, or immunoglobulin gene translocations, as discussed below.

Other Copy Number Aberrations

In addition to chromosomal rearrangements, a number of rare, but recurrent, copy number changes are present in the genome of CLL patients (Table 8.1). Interstitial deletions of 14q are rare and deletion break points are clustered and may juxtapose the immunoglobulin enhancer to an as-yet unidentified gene. Deletions of 6q can be detected in approximately

5% of patients with CLL, but multiple minimally deleted regions have been identified or candidate gene has been proposed. The clinical significance of 6q loss is uncertain but has been associated with atypical lymphocyte morphology-extensive lymphadenopathy but not chemoresistance. Overrepresentation of chromosome 2p has been consistently reported as a genomic abnormality in CLL in studies using CGH and SNP arrays. Overall occurrence has been reported in 5% of early-stage CLL patients, rising to 28% in stage B and C disease. Conventional cytogenetics shows a variety of mechanisms resulting in duplication. However, 2p duplication rarely occurs in isolation and is often associated with adverse genetic abnormalities: del(11q) and del(17p) and unmutated *IGHV* genes. The duplicated region most commonly reported includes the oncogenes *REL* and *MYCN*. Expression of NMYC has been shown to be elevated in the presence of a 2p24 duplication suggesting that this gene may be of significance in disease progression. Other regions targeted by recurrent copy number changes at a low incidence include duplications of 8q24, and deletions of 15q15.1 (4% of cases) and 3p21 (3% of cases), with *c-MYC*, *MGA*, and *SETD2* as candidate genes, respectively [30, 32].

Structural Chromosomal Rearrangements

Translocations involving the immunoglobulin gene loci are rare in CLL (<5%) and result in juxtaposition of a number of recurring partner genes to the transcriptional control of the immunoglobulin locus. The most common partner genes are *BCL2* (18q22) and *BCL3* (19q13) and more rarely *BCL11A* (2p15), *CCND3* (6p21), *CMYC* (8q24), and *CCND1* (11q13). The t(14;19)(q32;q13) is usually associated with trisomy 12 and most patients have atypical lymphocyte morphology, an atypical immunophenotype, and unmutated IGHV genes. The clinical course is usually progressive and the response to standard chemotherapy is poor. The t(11;14) translocation has been described as a rare secondary aberration in patients relapsing after therapy [64]. The clinical importance of the t(14;18) is uncertain, and it occurs as a primary or secondary event, commonly concomitant trisomy 12. The t(2;14)(p16;q32) is extremely rare and is associated with atypical morphology, immunophenotype, and bulky disease [67]. Translocations involving *CMYC* and a variety of partners including the immunoglobulin gene loci are associated with increased prolymphocytes, complex cytogenetic abnormalities, and a poor prognosis.

Patterns and Mechanisms of Genomic Complexity

Growing evidence suggests that the presence of genomic complexity [5, 65], defined by the presence of increased numbers of chromosomal lesions, can predict short overall survival [5, 65], independent of the presence of 17p deletions [66]. Indeed, interest has recently refocused on the presence of karyotypic complexity, defined by chromosomal banding analysis, as it represents a powerful independent predictor of poor response to the kinase inhibitor, ibrutinib [68].

As genomic complexity is often observed in patients with *ATM* and *TP53* gene lesion [69], it may be that these defects allow telomeres to shorten below the length at which apoptosis or senescence is normally triggered, thus leading to further telomere attrition and accumulation of short telomeres [70, 71], enabling uncapped telomeres to fuse, resulting in genomic instability. While DNA damage can be accumulated over time, high levels of DNA damage can also be acquired rapidly. One example is chromothripsis, a catastrophic process involving genome shattering that occurs during a single mitotic cycle resulting in a pattern of oscillating DNA copy number changes along a single chromosome, or a few chromosomes [67]. The frequency of chromothripsis is approximately 3–5% of human cancers, was first identified in a CLL patient [30, 67], and is the result of a partitioned chromosome(s) in a micronucleus that becomes damaged and is reintegrated into the daughter nuclei [72, 73]. Chromothripsis occurs preferentially in patients with unmutated *IGHV* genes and high-risk genomic aberrations [30], such as mutations in *TP53*, suggesting that a defective DNA damage response is critical to the process of chromothripsis, or the tolerance of the genomic damage [74]. While patients with chromothripsis exhibit both inferior OS and PFS, it is unclear if this is independent of the aforementioned poor-risk genomic lesions [30]. The acquisition of multiple single-nucleotide variants can also occur in a single mitotic explosion, termed kataegis [75], and has also been observed in the genome of CLL patients. This process drives cytosine-specific mutagenesis, often in regions flanking sites of genomic rearrangement, and can result in up to several thousand base-pair substitutions occurring rapidly [76].

In CLL, the biological and clinical importance of telomere structure has been well studied. Telomeres cap the ends of chromosomes, playing a crucial role in the maintenance of genomic integrity. Telomere length is a critical determining factor of telomere function, with critically eroded telomeres being subjected to aberrant DNA repair leading to telomere fusion and genomic instability. The presence of shortened telomere length (TL) is associated with poor clinical survival when assessed by a variety of techniques, as with terminal restriction fragment analysis, FISH-based approaches, and quantitative PCR, with a strong association between short TL and unmutated *IGHV* status [77–81]. The application of STELA (single telomere length analysis) to detect very short telomeres showed that shortening TL in CLL patients can result in loss of their end capping function and make them subject to telomere fusion [82]. Clinically, the acute telomere attrition can precede disease progression, providing further evidence that TL in asymptomatic disease may have powerful predictive value [83].

The Mutational Landscape of CLL

NGS technology has permitted the interrogation of the entire cancer genome for sequence changes at base-pair resolution. WGS and WES data has been collected on more than a thousand CLL patients [17, 18, 33, 34, 36, 38, 42, 84, 85]. However, papers from Landau et al. [17] and Puente et al. [38] published in 2015 provide the most comprehensive and current depiction of the mutational landscape of CLL. Based on the six possible base substitutions and information on the bases immediately 3′ and 5′ to the mutated base, 30 distinct mutational signatures have been identified across all cancer types [76]. Five different signatures have been recognized in CLL. Two (signatures 1 and 5) show a correlation between the number of mutations and increasing age, while two (signatures 2 and 13) have been attributed to activation of AID/POBEC cytidine deaminases. Signature 9 is confined to B-cell tumors that have undergone somatic hypermutation of variable region immunoglobulin genes and is attributed to DNA polymerase η-mediated repair of AID-induced lesions [76, 86]. Understanding underlying mutational mechanisms has clear clinical utility. Modifying the age-related mutational signatures may delay cancer initiation. Furthermore, the recognition of key signatures in asymptomatic patients may facilitate early diagnosis or quantify genotoxic exposure levels.

CLL cases have a median of 0.6–0.87 mutations per megabase (Mb) of genomic DNA which is low compared to solid tumors. Landau et al. and Puente et al. identified an average of 15.3 and 26.9 somatic mutations per patient, respectively [18, 38]. There is a level of discordance between the recurrently mutated genes identified by these two studies. These discrepancies probably reflect the relatively small cohort size and therefore consequent statistical power to identify rare mutated cancer genes, but may also be attributable to the different cohort composition, sequencing platforms, and bioinformatics pipelines used. For example, 22 recurrently mutated genes were implicated in both studies (including *BIRC3*, *CHD2*, *XPO1*, and *EGR2*), while genes only identified by a single study include *SETD2*, *ARID1A*, *NFKBIE* [38], *KRAS*, and *SAMHD1* [17]. While these two studies and indeed a plethora of others have failed to identify a unifying mutation in all patients, and it is unlikely that such a mutation exists at the genomic level, four genes are recurrently mutated at relatively high frequencies across multiple studies. In addition to the aforementioned genes, *TP53* and *ATM*, the other prevalent mutated genes are *NOTCH1* and *SF3B1* (Fig. 8.2). In addition, a glut of additional mutated genes occur at a frequency of approx. 5%, and lead to the dysfunction of eight key cellular processes: (1) cell cycle regulation, (2) DNA damage response, (3) apoptosis, (4) NOTCH1 signaling, (5) RNA metabolism, (6) NF-kB signaling, (7) chromatin remodeling, and (8) BCR signaling (54) (Tables 8.2 and 8.3, Fig. 8.3). These mutations can be present in the entire cancer cell population (clonal) or found in only a small "subclonal" population of cells. Deep sequencing approaches can detect low-level subclonal mutations present in as little as 0.3% of cancer cells [61] beyond the resolution of standard Sanger sequencing [89].

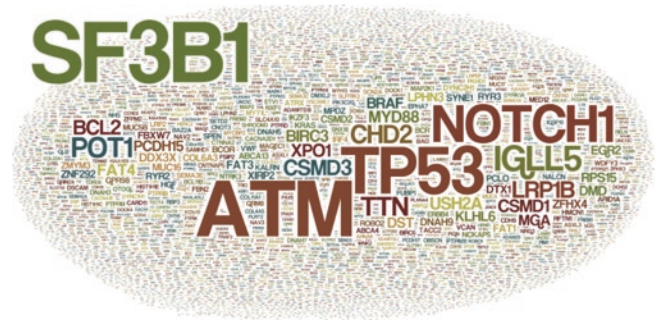

Fig. 8.2 Recurrently mutated cancer genes identified in CLL. This word cloud represents the presence and prevalence of gene mutations in CLL, where the font size represents the mutational frequency of a given gene. The mutation data was obtained from the Sanger Institute Catalogue of Somatic Mutations in Cancer web site, http://cancer.sanger.ac.uk/cosmic [87], with permission

Mutations of *NOTCH1* and Associated Proteins

In CLL, the importance of Notch signaling is well established where activation confers apoptosis resistance and cell survival in CLL cells [90] and Notch1-signaling inhibitors, such as γ-secretase inhibitors, accelerate B-CLL cell apoptosis by proteasome inhibition and endoplasmic reticulum stress enhancement [91]. The role that gene mutations play in activating Notch signaling and mutations was first described in a small cohort of 43 patients, where 2 patients (4.6%) harbored a heterozygous 2 bp frame-shift deletion (ΔCT7544–7545, P2515Rfs*4) within the PEST domain of *NOTCH1* [35]. The Spanish CLL Genome Consortium confirmed these early observations and demonstrated that this variant creates a premature stop codon, the removal of the PEST sequence from C-terminal protein domain that ultimately results in the accumulation of an active Notch1 isoform in tumor cells [42]. Subsequent studies show that mutations across exon 34 of *NOTCH1* [33, 34, 36, 42], and even within the 3′ UTR of the gene, where noncoding mutations create aberrant splicing events, result in the loss of the *NOTCH1* PEST domain, and consequent constitutive Notch1 activation [38]. *NOTCH1* mutations associate with unmutated *IGHV* CLL, expression of CD38 and ZAP-70, the presence of trisomy 12, short telomeres, and increased prevalence of prolymphocytes [81, 92, 93]. Mutation frequency varies enormously based on the stage of the disease analyzed, ranging from 3% in MBL and 6–10% at CLL diagnosis to >20%

Table 8.3 Recurrently mutated genes in CLL, without biological or clinical evidence

Gene name	Gene nomenclature	Approximate frequency (%)	References
FAT atypical cadherin 1	*FAT1*	10	[88]
Low-density lipoprotein receptor-related protein 1B	*LRP1B*	5	[34]
MGA, MAX dimerization protein	*MGA*	5	[17]
Zinc finger protein 292	*ZNF292*	5	[38]
Zinc finger, MYM-type 3	*ZMYM3*	4	[36]
DEAD (Asp-Glu-Ala-Asp) box polypeptide 3, X-linked	*DDX3X*	3	[18, 36]
Mitogen-activated protein kinase 1	*MAPK1*	3	[36]
IKAROS family zinc finger 3	*IKZF3*	3	[17]
Bromodomain adjacent to zinc finger domain 2A	*BAZ2A*	3	[17]
Histone cluster 1, H1e	*HIST1H1E*	3	[18]
BCL6 corepressor	*BCOR*	3	[18, 38]
Receptor (TNFRSF)-interacting serine-threonine kinase 1	*RIPK1*	3	[18]
Caspase recruitment domain family member 11	*CARD11*	3	[17]
Kelch-like family member 6	*KLHL6*	2	[42]
Nuclear RNA export factor 1	*NXF1*	2	[17, 38]
Nuclear factor kappa B subunit 2	*NFKB2*	2	[38]
Cyclin D2	*CCND2*	2	[38]
Spectrin repeat containing nuclear envelope protein 1	*SYNE1*	2	[38]
AT-rich interaction domain 1A	*ARID1A*	2	[38]
CCR4-NOT transcription complex subunit 3	*CNOT3*	2	[38]
Dual-specificity tyrosine phosphorylation-regulated kinase 1A	*DYRK1A*	2	[17]
Mitogen-activated protein kinase kinase 1	*MAP2K1*	2	[17]
Inositol-trisphosphate 3-kinase B	*ITPKB*	2	[18]
TNF receptor-associated factor 2	*TRAF2*	2	[17]
Far upstream element-binding protein 1	*FUBP1*	1.9	[17]
Exportin 4	*XPO4*	1.8	[17]
Neuroblastoma RAS viral (v-ras) oncogene homolog	*NRAS*	1–3	[18, 45]
Protein tyrosine phosphatase, non-receptor type 11	*PTPN11*	1	[17, 38]
SET domain containing 1A	*SETD1A*	1	[38]
E74 like ETS transcription factor 4	*ELF4*	1	[17]
BRCA1/BRCA2-containing complex subunit 3	*BRCC3*	1	[17]
EWS RNA-binding protein 1	*EWSR1*	1	[17]
ATRX, chromatin remodeler	*ATRX*	1	[38]
Family with sequence similarity 50 member A	*FAM50A*	1	[17]
TNF receptor-associated factor 3	*TRAF3*	1	[17, 38]
Additional sex combs like 1, transcriptional regulator	*ASXL1*	1	[17, 38]
Checkpoint kinase 2	*CHEK2*	1	[17]
G protein subunit beta 1	*GNB1*	1	[17]
Histone cluster 1 H1 family member b	*HIST1H1B*	1	[17]
Pim-1 proto-oncogene, serine/threonine kinase	*PIM1*	1	[17]
Retinoblastoma 1	*RB1*	2[a]	[24]

[a]Frequency in those patients with large 13q deletions which include the *RB1* locus. Defined as "type II" deletions [55]

of patients with alkylating agent or purine analogue-refractory disease [33, 94]. An additional 3% of CLL patients harbor the noncoding 3′UTR mutation [38, 62, 95]. Patients with a mutant *NOTCH1* have inferior survival compared to wild-type patients, compared to 11q deleted patients [16, 92]. The mutational frequency of *NOTCH1* is higher in CLL lymph nodes than in matched peripheral CLL B-cells (24%) and the Notch pathway is frequently activated in lymph node cases independently of *NOTCH1* mutational status, suggesting the existence of other initiating mechanisms, such as ligand activation [96]. Functionally, microenvironmental interactions appear to be required for Notch activation in mutated cases, where these interactions foster conditions that may favor drug resistance [97], but may be overcome with the use of γ-secretase inhibitors, particularly in combination with fludarabine [98]. Mutations in other Notch signaling

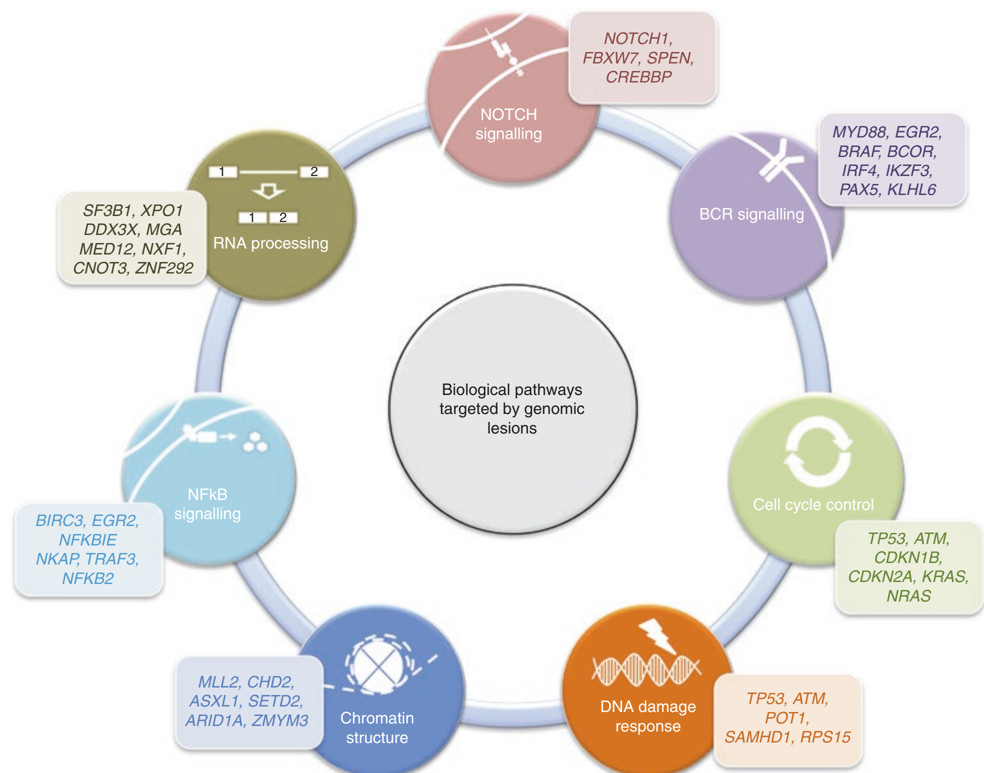

Fig. 8.3 The biological pathways deregulated in CLL by somatic gene mutations. The key biologically relevant genes recurrently mutated in CLL, and the pathways and processes to which they contribute. Genes with functional diversity are represented in the pathway to which they most significantly contribute

proteins have been identified in CLL. For example, *FBXW7*, which targets activated *NOTCH1* for degradation, is mutated in ~2.5% of patients and may provide another mechanism for activated Notch signaling [99].

SF3B1 Mutations and RNA Processing

SF3B1, an important component of the RNA splicing machinery that achieves successful transcription and guarantees the functional diversity of protein species using alternative splicing, is recurrently mutated in CLL but not in other chronic B-cell lymphoproliferative neoplasms [34, 36, 85]. 5–17% of CLL patients harbor *SF3B1* mutations where they are associated with advanced clinical stage, 11q23 deletions, presence of stereotyped IGHV usage (subset #2), and fludarabine-refractory disease in cases with no *TP53* abnormality. Furthermore, they predict reduced TFTT and OS independent of other prognostic variables [34, 36, 85, 99–101].

Using targeted and global experimental approaches, early studies showed that *SF3B1* mutations result in aberrant splicing [34, 36] that is driven by the use of a different branch point sequence in mutant *SF3B1* patients [102]. Initial studies identified a highly expressed truncated *FOXP1* transcript, *FOXP1w* in *SF3B1*-mutated CLL, that lacks two putative PEST domain sequences involved in protein degradation [34]. A subsequent RNA-Seq study implicated an aberrant spliced *ATM* transcript [103] and it is now evident that *SF3B1* mutations may impact DNA damage response [104]. Aberrant splicing of genes involved in B-cell differentiation, Hippo signaling, and NF-κB activation has also been implicated in *SF3B1*-mutated CLL [103]. The identification of mutations within the spliceosome complexes raises the possibility that CLL tumor cells may be sensitive to spliceosome inhibitors. Indeed, *SF3B1* mutations confer sensitivity to the splicing modulator sudemycin, promoting an anti-tumor effect with the BTK inhibitor, ibrutinib [105], and the SF3B1 inhibitor spliceostatin A induces cell death in CLL cells through Mcl-1 downregulation, most markedly in combination with Bcl-2/Bcl-XL antagonists [106].

Another gene involved in RNA processing has also been implicated in CLL pathogenesis, *XPO1*, which encodes the nuclear exporter, Exportin-1, responsible for controlling the directional movement of 100 s proteins and RNA species from the nucleus to the cytoplasm. 4.6% of *IGHV*-unmutated CLL cases harbor somatic mutations in *XPO1* [42], and cause increased Exportin-1 levels in tumor cells, and consequent externalization of key TSP, compromising a cell's ability to respond to DNA damage [107]. Inhibitors of XPO1 may restore apoptotic pathways and chemosensitivity in CLL cells by facilitating nuclear export of key proteins [107, 108].

Other Mutations

Other recurrently mutated genes have emerged, and are continuing to emerge, from high-throughput sequencing projects, albeit at low frequencies (Tables 8.2 and 8.3) [18, 36, 42].

As previously noted, these genes often act within key biological pathways (Fig. 8.3) [18, 34, 36]. Some of the most established are outlined below:

1. **NFKB Signaling**. Recurrently mutated genes have been identified in both the canonical and noncanonical NFκB signaling pathways, where they can result in activation signaling [37, 56, 109, 110]. In addition to aforementioned involvement of the *BIRC3* gene, up to 10% of CLL cases have mutations targeting *NFKBIE*, which encodes NFκB inhibitor epsilon (IKBE). Cellular studies show reduced IKBE expression in mutant cases, decreased IKBE-p65 interactions and increased nuclear p65 levels, and constitutive NFκB activity [110].
2. **B-cell Receptor Signaling.** *MYD88*, a crucial adaptor of the Toll-like receptor (TLR) complex, is mutated in approx. 5% of CLL cases, occurring exclusively in patients with mutated *IGHV* genes. Upon TLR ligand binding, a homodimer of MYD88 is recruited to the receptor, forms a complex with IRAK4, activates IRAK1 and 2, and ultimately leads to TRAF6 activation, phosphorylation of IκBα, and activation of NF-κB [111]. In CLL, the L265P mutation provides constitutive activation of NF-κB activity, by imposing MYD88-IRAK signaling even when ligand receptor binding is absent [42]. However, the clinical impact of MYD88 mutations remains controversial, as conclusive evidence demonstrating independent prognostic significance is lacking [112, 113]. The transcription factor, *EGR2*, is activated by B-cell stimulation and also mutated in 8% of advanced-stage CLL. *BRAF* mutations have a postulated role in fludarabine sensitivity and are associated with reduced time to first treatment [37, 38, 114].
3. **Cell cycle, Apoptosis, DNA Damage.** In addition to the well-studied involvement of the *TP53* and *ATM* loci, other genes with a role in these processes have been recently implicated. *RPS15,* which encodes a component of the 40S ribosomal subunit, is mutated in 20% of CLL patients relapsing post-chemo-immunotherapy [39]. *RPS15* mutations are early clonal events, associated with reduced survival, the functional consequences of which are defective p53 stability and increased degradation [39]. Mutations in the nuclease *SAMHD1* are reported in 3% of patients at diagnosis and are enriched in therapy-refractory patients [43]. Preliminary data suggests that mutations may promote tumorigenesis by deregulation of DNA repair [43]. *POT1*, a component of the shelterin complex, plays a critical role in the protection of telomeres. At diagnosis, mutations occur in approx. 3% of patients [84, 115] rising to 8.1% in patients receiving chlorambucil-based therapy, where they are associated with a shorter survival [46]. In *POT1*-mutated cell lines, chromosomes become fragile, with numerous telomeric and structural aberrations implicating these mutations in promoting genomic instability [84]; this association is not supported by the study of primary CLL tumors [115].
4. **Chromatin Modifiers.** *CHD2*, a chromatin modifier, is mutated in 5.3% of patients, principally in *IGHV*-mutated tumors. Mutations are principally truncating or target functional domains and functional experiments demonstrate alteration of the nuclear distribution of *CHD2* and protein association with actively transcribed genes in mutated patients [116]. Histone methyltransferases (HMTs) are essential epigenetic regulators of chromatin modification and recurrent mutations targeting such genes have only recently been documented in CLL [38]. *SETD2*, the histone methyltransferase non-redundantly responsible for the trimethylation of lysine 36 on histone 3 (H3K36me3), is mutated in up to 4% of patients, and likely to be an early loss-of-function event associated with aggressive disease [32].
5. **Noncoding mutations.** In addition to the aforementioned *NOTCH1* 3′UTR mutations, a second recurrent noncoding mutation has been reported, resulting in deregulation of *PAX5*. In CLL, mutations located within a telomeric enhancer element, 330Kb from the *PAX5* locus, result in reduced *PAX5* expression, and were the only recurrent mutation in a subset of IGHV-unmutated CLL patients, suggesting that these mutations may contribute to disease pathophysiology [38].

Clonal Evolution

Historically, we often consider genomic heterogeneity to exist between patients (inter-tumor), in part explaining the biological and clinical variability that exists between individuals with the same disease. However, it is evident that considerable "intra-tumoral" heterogeneity also exists, such that a tumor can contain many genetically and biologically unique subclonal populations of cancer cells. This cellular plasticity is a prerequisite for Darwinian selection that ultimately selects cellular populations with favorable biological traits, driven by the pressure of therapy and even the tumor microenvironment. In CLL, researchers working with FISH and SNP arrays have long observed the acquisition of genomic aberrations during disease course, with the suggestion that the diagnostic and relapse disease can be genetically distinct [117–120]. However, it has been the application of NGS to temporally and even anatomically discrete cancer specimens from the same CLL patient that has conclusively revealed the composition of clonal expansion [121]. Clonal evolution can occur quickly, or over a more protracted period of time, and follows two simple evolutionary models [122]. Firstly, tumor evolution can develop in a linear fashion, with the maintenance of a founder clone with successive acquisition of new mutations. Secondly, competition between different cancer subclones can persist, resulting in a more

complex branching anatomy. In the context of a branching evolution, convergence can occur, where independent mutations in the same genes can be acquired in different subclones [123].

Landau and coauthors [18] showed that passenger events accumulate before the acquisition of recurrent driver mutations (e.g., del(13q), tri12, and *MYD88* mutations) with subsequent malignant initiation. During CLL progression, later subclonal driver mutations target cancer genes like *ATM*, *TP53*, and *RAS* and undergo clonal expansion. The authors also showed an elevated prevalence of clonal evolution in treated patients, and linked the presence of subclones to adverse survival [18]. Subsequent studies demonstrated genomic heterogeneity between different anatomical compartments with mutations expanding in the lymph node and repopulating the peripheral blood compartment at relapse following positive selection by therapy [122, 124, 125]. Rose-Zerilli et al. reported a CLL patient whom at diagnosis presented with mutated immunoglobulin genes, but who developed a fatal *IGHV*-unmutated CLL clone years later, not evident at diagnosis with traditional molecular approaches, but detectable with modern NGS approaches. These observations have important clinical implications and support a model in which low-level subclonal mutations present in early-stage disease can anticipate the evolutionary course of the disease [17].

Epigenetic Abnormalities

There is increasing evidence that epigenetic abnormalities are both frequent and important in the initiation and progression of human malignancies, including CLL. Epigenetic mechanisms that are deregulated in cancer include changes to higher order chromatin structure, such as histone modifications, and DNA methylation, both of which are crucial layers of epigenetic programming that regulates gene transcription and genome stability, and contribute to normal B-cell development. DNA methylation, which occurs predominantly at the cytosine residue of CpG dinucleotides, is the most well-studied epigenetic mechanism in CLL [19, 20, 126, 127]. Candidate gene approaches identified differential methylation at promoters and CpG islands of genes including *DAPK1*, TCL1, *ZAP70*, *HoxA4, TWIST2*, as well as CLL-associated microRNAs and long intervening noncoding RNAs [128, 129]. Although DNA methylation and gene expression are frequently poorly correlated, a strong association was noted between the methylation status of *ZAP70*. The methylation status of a single ZAP70 CpG site was subsequently shown to have prognostic significance [130, 131].

New insights have emerged from global methylation profiling of normal B-cell subsets and large CLL cohorts, using both methylation arrays and whole-genome bisulfite sequencing [19, 20, 126, 132]. Normal B-cell maturation from naive to memory B-cells is accompanied by prominent hypomethylation, especially of enhancer and promoter regions and gene bodies together with hypermethylation in regions of transcriptional elongation [20, 132]. The methylation profile of CLL closely recapitulates that seen in normal B cells, such that IGHV-mutated and unmutated CLL maintain the epigenetic signature of memory B-cells (MBC) and naive B-cells (NBC), respectively. Interestingly, both studies identified a third epigenetic CLL subgroup with an intermediate methylation signature enriched for M-CLL with fewer somatic IGHV mutations [126]. These three CLL epitypes exhibit different clinico-biological features, with the MBC-like CLL cases exhibiting a more indolent clinical course. As a result of these studies, an assay has been developed to assess the methylation status of 5 CpG dinucleotides that can identify these three prognostically relevant groups in a simple manner [127].

Despite the similarity of CLL methylation signatures to their likely cells of origin, aberrant methylation is also observed, for example in the binding sites for key transcription factor families [20]. The mechanisms underlying aberrant methylation and its importance in the pathogenesis of CLL are under investigation. Although initial observations showed that global DNA methylation tends to remain temporally stable in sequential tumor samples [133], more recent studies have identified intra-tumor epigenetic heterogeneity. This is associated with subclonal genomic heterogeneity and an adverse outcome [19, 126].

Genetic Predisposition to CLL

Epidemiological surveys have shown that CLL has one of the highest familial risks of any cancer, with an 8.5-fold increased risk among first-degree relatives for developing CLL and a 1.9-fold risk for other B-cell chronic lymphoproliferative disorders, especially lymphoplasmacytic lymphoma and hairy cell leukemia [134, 135]. Genome-wide association studies undertaken in over 5000 patients with CLL and 12,000 controls have identified over 30 loci associated with increased susceptibility to CLL [136–140]. The susceptibility to CLL associated with each variant single-nucleotide polymorphism (SNP) is low, but increases if multiple risk variants are co-inherited. Most SNPs map to noncoding regions of the genome, frequently close to or within genes involved in apoptosis, B-cell differentiation, or telomere function suggesting that they may act by influencing the expression of these genes. This mechanism was recently confirmed at the susceptibility locus located at 15q15.1. A SNP (rs539846) within this locus maps to a super-enhancer in intron 3 of the B-cell lymphoma 2 modifying factor gene (*BMF*), a pro-apoptotic BH3 protein which binds to, and neutralizes, the anti-apoptotic protein BCL2. The rs539846 risk allele modifies a transcription binding site

for the transcription factor RELA, reducing BMF expression and potentially attenuating the apoptotic response [141].

Although the somatically acquired genomic mutations associated with sporadic CLL have rarely been implicated in genetic susceptibility to CLL, inactivating germline mutations in shelterin genes, especially POT1, critical for telomere function, have recently been discovered in CLL patients with strong family histories of CLL, consistent with the GWAS data. The authors estimated that 11% of familial CLL may be ascribed to mutations in this class of genes [142].

The Molecular Pathogenesis of Richter's Syndrome

Richter's syndrome (RS) denotes the histological diagnosis of either diffuse large B-cell lymphoma (DLBCL) or more rarely Hodgkin's lymphoma in a patient known to have CLL. In most cases the lymphoma is clonally related to the CLL but clonally unrelated lymphomas are also well documented. Preliminary investigations into the molecular mechanism of transformation to clonally related DLBCL have been performed [143, 144], but considerably less is currently known about the transformation process of other clonally related lymphoma subtypes or in patients who transform to clonally unrelated DLBCL.

In clonally related transformed DLBCL, the BCR is likely to play an important role in the transformation process, as clonally related DLBCL cases show remarkable bias in BCR usage, with enrichment of stereotyped "subset 8" cases (Fig. 8.4). This suggests that a restricted panel of antigenic epitopes are important in the transformation process. Deletions and/or mutations of the TP53 genes are the most prevalent genetic lesions found in clonally related DLBCL, identified in approx. 60% of cases [143]. The chemo-refractory phenotype that predominates in this disease is likely to be determined by loss of *TP53* function, and the resultant loss of cell cycle control that mediates resistance to chemotherapeutic agents. Approximately 40% of cases with clonally related DLBCL transformation show evidence of *MYC* aberrations, often acquired at transformation [143, 144]. In 30% of cases, *MYC* is targeted by structural rearrangements, including juxtaposition of *MYC* to the *IGHV* locus, genomic gain of 8q24, or activating point mutations [143]. Mutually exclusive to *MYC* abnormalities are activating mutations within the PEST domain of *NOTCH1* occurring in approximately 30% of cases, resulting in constitutively activated Notch signaling. While rare in diagnostic CLL cohorts, *CDKN2A* deletions can be identified in 30% of DLBCL transformed patients, and are likely to emerge in the tumor clone at transformation [143, 144].

Risk factors for the acquisition of a clonally related DLCBL principally focus on CLL cases with "subset 8" IGHV usage [145], who have a very high risk of transformation, approximately 80% at 10 years, and those that harbor a *NOTCH1* mutation; *NOTCH1*-mutated CLL cases have a cumulative probability of Richter transformation of 45% (compared to 4% for wild-type *NOTCH1* cases) [146]. Post-transformation, patients lacking TP53 lesions display more

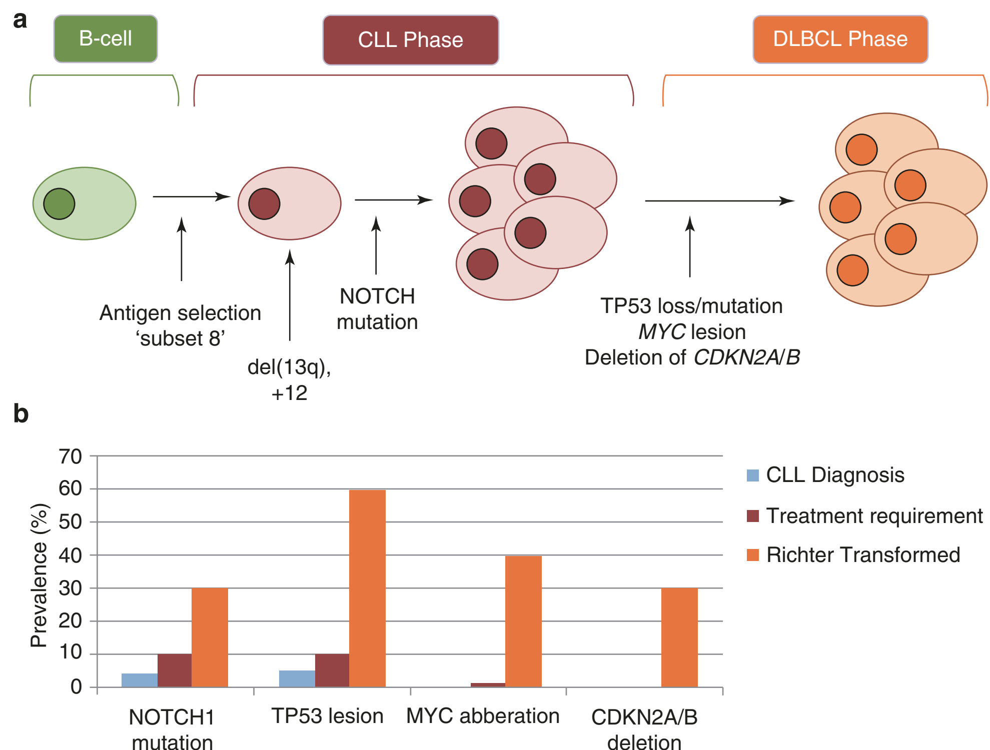

Fig. 8.4 The molecular pathogenesis of Richter's syndrome. (**a**) shows the accumulation of genomic lesions through the process of CLL development, progression, and transformation. (**b**) shows the prevalence of key genomic aberrations through the process of CLL development, progression, and transformation that are causally implicated in Richter transformation

favorable outcome than TP53-deleted or -mutated cases [147]. The changing therapeutic landscape for CLL will likely mold the epidemiology of transformation, as the non-genotoxic nature of modern treatment modalities will exert a different selective pressure on the tumor clone. Given the high prevalence of *TP53* lesions in transformed DLBCL, the prediction is that fewer *TP53*-driven transformation events will occur. However, it will be important to study transformation mechanisms in the context of new therapies, as clonal selection will sculpture the molecular mechanisms of transformation. Using targeted therapeutics against the molecular pathways that are disrupted in transformed CLL may show promise in the management of these tumors, such as the BCR, gamma secretase, bromodomain and cyclin-dependent kinase inhibitors, and novel compounds gaining traction for the management of aggressive lymphoma, such as immune checkpoint and XPO1 inhibitors.

Genomic Abnormalities in Clinical Practice

Genomic abnormalities are one of an increasing number of biomarkers that have clinical relevance in the management of patients with CLL. Although the diagnosis of CLL is based on immunophenotypic and morphological criteria, genomic abnormalities have both prognostic value in calculating the natural history of CLL patients and predictive value in determining the response to therapy. Furthermore, genomic screening can identify mechanisms of drug resistance and finally genomic abnormalities may act as therapeutic targets. Table 8.4 lists examples of the genomic abnormalities reported to have adverse prognostic significance in CLL in either large patient cohorts or clinical trials.

Predicting Natural History

Over 80% of CLL patients are asymptomatic at diagnosis and have a low tumor burden. Precisely identifying those likely to progress has the potential to inform patients and their families, and influence the need for and timing of follow-up and the timing and selection of optimal treatment. Following the recognition that individual copy number abnormalities, genomic mutations, and genomic complexity could influence both time to first treatment (TTFT) and overall survival when measured at or close to diagnosis, a variety of models have been developed which encompass the most frequent genomic abnormalities alone or in combination with other biomarkers.

A large study by Davide Rossi and colleagues has proposed a prognostic algorithm for overall survival through the integration of gene mutations and chromosomal abnormalities [16]. The authors analyzed CLL patients for FISH abnormalities and sequence mutations and offered four

Table 8.4 Genome lesions of clinical importance

Genomic abnormality	Clinical context	TTFT	PFS	OS	References
Genomic complexity (GC)	At diagnosis	X			[148, 149]
	Chemo-immunotherapy trial			X	[46]
	Ibrutinib for R/R disease			X	[68]
Chromothripsis	Chemo-immunotherapy trial		X	X	[30]
del(11q)	At diagnosis				[25]
	CT trial		X	X	[150]
TP53 abnormalities	At diagnosis	X		X	[6, 13]
	CT, Chemo-immunotherapy trials		X	X	[150–152]
	Chemo-immunotherapy for relapse/ refractory disease			X	[153, 154]
	Ibrutinib trial for previously untreated or relapsed disease		X		[155]
Subclonal drivers	Pre-chemo-immunotherapy (all drivers)	X	X		[17, 18]
	TP53 pretreatment	X		X	[61, 62]
Multiple drivers	Pretreatment	X			[38]
SF3B1	At diagnosis	X			[156]
	Chemotherapy trial			X	[92]
	Chemo-immunotherapy trial		X	X	[153]
NOTCH1	At diagnosis	X			[156]
	Chemotherapy trial			X	[92]
	Chemo-immunotherapy		X	X	[153]
RPS15	Chemo-immunotherapy trial		X		[17]

Time to first treatment (TTFT), progression-free (PFS) and overall survival

risk classifications: (1) high-risk patients with either *TP53* defects and/or *BIRC3* disruption; (2) intermediate-risk, harboring *NOTCH1* and/or *SF3B1* mutations and/or del(11q); (3) low-risk, harboring trisomy 12 or a normal profile; and perhaps most importantly (4) a very-low-risk group with del(13q) only, whose survival did not differ from that of a matched general population. This model added significantly to the "Döhner" model [157], due to the coexistence of poor-risk gene mutations in low-risk groups defined purely based on FISH and retained prognostic significance over time regardless of clonal evolution. However subsequent similar large studies which also used TTFT as an endpoint have failed to completely replicate these findings, highlighting the difficulty of developing models that are sufficiently precise for routine use and the need for even larger, well-designed prospective studies [99, 112].

A recently introduced international prognostic index (CLL-IPI) performed multivariate analysis of 27 clinical and biological factors including 13q, 11q, 6q, and 17p loss; trisomy 12; and mutations of *TP53*, *SF3B1*, and *NOTCH1* on a large cohort of patients predominantly entered into first-line chemotherapy or chemo-immunotherapy trials. Five prognostic factors were identified: *TP53* status, *IGHV* mutational status, serum B2 microglobulin, clinical stage, and age, from which a prognostic index was derived identifying four risk groups with different overall survivals [158]. The CLL-IPI has subsequently been shown to predict TTFT in early-stage CLL [159].

Predicting Outcome Following Treatment

TP53 abnormalities. As outlined earlier in this chapter, copy number abnormalities such as del(11q) and deletion of *SETD2* and genomic mutations of *SF3B2, NOTCH1, BIRC3, SAMHD1*, *RPS15*, and *EGR2* appear to influence the outcome of patients receiving chemotherapy or chemo-immunotherapy. However the effect is either modest or has not been evaluated in multivariate analyses of large clinical trials. As a consequence the only genomic abnormalities that determine the choice of therapy in routine clinical practice are deletion and/or mutation of *TP53*, and current guidelines recommend screening for TP53 abnormalities prior to first-line and relapse therapy. Their presence is unequivocally linked to a dismal response to standard chemo-immunotherapy and is an indication for novel agents such as signaling inhibitors or pro-apoptotic BH3 mimetics acting through TP53-independant mechanisms. Conversely the absence of del(17p) or del(11q) in patients with mutated *IGHV* genes identifies patients with long overall survival following standard chemo-immunotherapy for fit patients (FCR) [160].

Although evidence for the predictive value of *TP53* abnormalities is overwhelming, the outcome of patients with these abnormalities is not uniform and is influenced by coexisting genomic abnormalities, immunogenetic status, and treatment type. As previously mentioned, it is well recognized that a small subset of early-stage patients with TP53 abnormalities may pursue a stable clinical course [161]. A more recent study of 69 patients with >10% TP53 loss using WES and SNP arrays to detect genomic mutations and CNAs, respectively, showed that the poorest outcomes were associated with bi-allelic TP53 abnormalities; clonal TP53 mutations; deletions of 3p, 4p, or 8p; and genomic complexity. Conversely cases that remained untreated for 5 years were enriched for mutated IGHV genes, subclonal or no TP53 mutations, and fewer CNAs [59]. Similarly, Guieze et al. noted that the poorest outcome of patients who received salvage therapy, having failed to respond to or relapsing after chemo-immunotherapy, had mutations of *TP53, ATM*, and *SF3B1* [162].

The impact of *TP53* abnormalities on treatments that act through *TP53*-independent mechanisms is mixed. *TP53* status does not influence the outcome of patients receiving an allogeneic stem cell transplant whereas the efficacy of ibrutinib in patients with a *TP53* abnormality is much greater than chemo-immunotherapy but inferior to *TP53* wild-type cases. This implies that *TP53* abnormalities may have an indirect effect on facilitating ibrutinib resistance possibly related to increased genomic instability.

NOTCH1 mutations. An interesting observation that emerged from genomic analysis of the German Study Group CLL8 trial was that the outcome of patients in the chemo-immunotherapy arm (FCR) was superior to those in the chemotherapy arm (FC) apart from those with *NOTCH1* mutations [153]. A similar phenomenon was observed in a trial of chlorambucil with or without the anti-CD20 antibody, ofatumumab, suggesting that NOTCH1 mutations may be a predictive factor for reduced benefit from chemotherapy and anti-CD20 antibody combinations [163].

Identifying Resistance Mechanisms

TP53 abnormalities account for primary or acquired resistance to chemotherapy-containing regimens in at least 50% of cases, reflecting the importance of an intact DNA damage response pathway for the activity of these agents. Unsurprisingly acquired resistance is encountered in a variety of hematological malignancies treated with signaling inhibitors or BH3 mimetics, and in CLL the mechanisms of resistance to ibrutinib have been partially elucidated. Whole exomic sequencing of tumor samples from six patients who relapsed during ibrutinib therapy identified a cysteine-to-serine mutation (C451S) in Bruton's tyrosine kinase (BTK). This reduced the binding affinity of ibrutinib for BTK, resulting in only transient, rather than the usual irreversible,

inhibition of *BTK*. In addition three distinct gain-of-function mutations in *PLCG2*, the kinase immediately downstream of BTK, were found in two patients [164, 165]. A subsequent study used whole exomic and deep sequencing in serial samples from five patients who relapsed after an initial partial response to ibrutinib. At relapse, one patient had a *BTK* C4815 mutation and one had multiple *PLCG2* mutations. The remaining three cases showed expansion of a preexisting clone harboring del(8p) with additional driver mutations. Deletion of 8p resulted in halpoinsufficiency of the TRAIL receptor and the authors provided functional data to suggest that this may be a resistance mechanism [166].

A key question is whether *BTK* and *PLCG2* mutations can also be detected either prior to BTK administration or during BTK therapy before clinical relapse and therefore act as a predictive marker. Although computational analysis indicates that very small *BTK* mutant clones are present pretreatment, these have not been identified in deep sequencing studies [166, 167].

Summary

Since the previous edition of this book, our understanding of the CLL genome has been transformed by modern technology. New driver mutations have been discovered and it can be safely predicted that all clonal and subclonal genomic abnormalities that impact the survival and proliferation of tumor cells in at least 1% of CLL cases will be identified. It can also be anticipated that single platforms that robustly and affordably identify all common drivers will become available. Furthermore, mechanisms underlying genomic instability and phenotypic consequences of both single and multiple genomic abnormalities and their interaction with environmental factors are becoming increasingly understood.

These advances coincide with a revolution in treatment of CLL brought about by the introduction of novel small molecules and immune therapies which are highly effective, frequently easier to administer, and better tolerated than previous chemotherapy regimens. Importantly, the treatments that target key signaling and apoptosis pathways retain activity in cases that acquire the common genomic abnormalities including those affecting DNA repair.

Given the trend towards precision medicine, it is pertinent to ask what impact genomic advances might have in an era of increasingly effective therapies. It is probable that the genomic basis of familial CLL will be sufficiently understood to justify screening of high-risk individuals. The presence of key genomic lesions may have utility for the differential diagnosis of mature B-cell malignancies in general. It is also highly likely that screening for genomic abnormalities will contribute further to the recognition of early-stage patients at risk of disease progression who might benefit from early therapy prior to clonal evolution and expansion. It remains to be seen whether combinations of highly active agents targeting different pathways will be curative regardless of the genomic landscape, but should resistant clones emerge, then next-generation sequencing will be instrumental in determining resistance mechanisms and the choice of alternative therapies.

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9 Treatment of Chronic Lymphocytic Leukemia and Related Disorders

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Burden of CLL

According to 2016 SEER data, there will be 18,960 new cases of CLL diagnosed and more than 100,000 people with CLL living in the USA [1]. The median age at diagnosis for chronic lymphocytic leukemia was 71 years and age-adjusted incidence rate was 4.6 per 100,000 men and women per year.

Diagnosis of CLL

The diagnosis requires peripheral blood clonal B lymphocytosis of ≥5 × 10^9/L persisting for at least 3 months [2]. The clonality of the cells needs to be confirmed by flow cytometry-based immunophenotyping demonstrating aberrant expression of the T-cell antigen CD5 along with B-cell antigens CD19, CD20, and CD23, and also by restriction of light-chain expression to either kappa or lambda (Table 9.1). The percentage of prolymphocytes in the peripheral blood may be up to 55%. Any excess of prolymphocytes will favor the diagnosis of B-cell prolymphocytic leukemia (B-PLL). Demonstration of cytopenias along with marrow infiltration by typical CLL cells is adequate for the diagnosis of CLL irrespective of the degree of lymphocytosis.

Small lymphocytic lymphoma (SLL) is a disease restricted to lymph nodes and lymphocytosis of <5 × 10^9/L.

"Monoclonal B lymphocytosis" (MBL) is a disease comprised of clonal B-cell lymphocytosis <5 × 10^9/L in the absence of lymph node involvement, disease-related symptoms, and cytopenias. The prevalence of MBL can be 4–5% [3, 4] among the general population over the age of 40 years. In a prospective study [3], 15% of subjects with MBL and lymphocytosis developed CLL after a median follow-up of 6.7 years. The absolute lymphocyte count correlated with progression to CLL.

Table 9.1 Criteria for diagnosis of CLL

	Working groups: NCIWG/IWCLL [2]
Parameter	
Diagnosis	
Lymphocytes (×10^9/L)	>5; ≥1 B-cell marker (CD19, CD20, CD23) + CD5
Atypical cells (%) (e.g. prolymphocytes)	<55
Duration of lymphocytosis	At least 3 months

NCIWG National Cancer Institute Working Group; *IWCLL* International Workshop on CLL

Table 9.2 Immunophenotypic analysis in chronic B-cell disorders

Disease	Sig	CD5	CD23	FMC7	CD22	CD79b
CLL	Weak	++	++	−/+	Weak/−	Weak/−
B-PLL	Strong	−/+	−	++	+	++
HCL	Strong	−	−	++	++	+
SL VL	Strong	−/+	−/+	++	++	++
FL	Strong	−/+	−/+	++	++	++
MCL	Strong	++	−/+	++	++	++

CLL chronic lymphocytic leukemia, *B-PLL* B-cell prolymphocytic leukemia

Modified from Matutes E, Owusu-Ankomah K, Morilla R, et al. The immunological profile of B-cell disorders and proposal of a scoring system for the diagnosis of CLL. Leukemia. 1994;8:1640–5

The differential diagnosis of CLL includes several other B-cell disorders. Flow cytometric analysis is critical for differentiating between CLL and other B-cell disorders (Table 9.2). A flow cytometry-based scoring system has been useful in situations where the diagnosis is not straightforward [5].

Clinical Features

At diagnosis, most patients are older than 70 years, with more than 95% over 45 years. The diagnosis of CLL is often incidental; routine blood counts may reveal an elevated absolute

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lymphocyte count (ALC). In symptomatic patients, fatigue and infections may be presenting features; B symptoms are rare. A smaller percentage of patients may present with autoimmune hemolytic anemia (AIHA) or autoimmune thrombocytopenia (AIT). Physical examination may reveal cervical, axillary, and/or inguinal lymphadenopathy. Splenomegaly is not uncommon. The majority of patients with CLL will develop hypogammaglobulinemia in their disease course [6, 7]; impaired cellular immunity is evidenced by lack of response to skin testing with tuberculin, candida, and other antigens. These immune defects predispose patients to recurrent infections [8].

Staging

The well-described staging systems for CLL include those of Rai [9] and Binet [10] staging (Table 9.3). The original Rai staging defined five stages from 0 to 4; this has been modified [11] to three stages by defining Rai stage 0 as low-risk group, stage 1 and 2 together designated as an intermediate-risk group, and joining stage 3 and 4 to form a high-risk group with a median survival of >12.5, 7, and 1.5 years for each risk group, respectively. Similarly in Binet stages A, B, and C median survivals are >10, 6, and 2 years, respectively [10]. Both these clinical staging systems are based on physical examination and routine blood counts and do not require radiological imaging. The value of computed tomography (CT) in early-stage CLL is not established.

Workup at Diagnosis

A physical examination that includes examination of the lymph nodes, routine blood counts, and peripheral blood flow cytometry to establish clonal B-cell lymphocytosis is sufficient to establish the diagnosis of CLL. Though the type of bone marrow infiltration (diffuse vs. nondiffuse) may carry prognostic information, the need for a bone marrow aspiration and biopsy can be replaced by new prognostic markers. FISH is required prior to any line of therapy given that therapy in patients with CLL with del(17p) may differ from first-line therapy for patients with CLL without del(17p).

Prognostic Factors

Clinical Prognostic Factors

In a randomized trial of chlorambucil versus observation in Binet stage A patients [12, 13], a subgroup of patients (designated "A") with hemoglobin ≥12 g/dL, lymphocyte count $<30 \times 10^9$/L, and fewer than 80% lymphocytes in the bone marrow aspirate was identified and had an overall survival (OS) comparable to an age-matched French population. Similarly, a lymphocyte doubling time of >12 months, Rai stage 0 disease, nondiffuse bone marrow pattern, hemoglobin ≥13 g/dL, and absolute lymphocyte count $<30 \times 10^9$/L define a group of "smoldering CLL" with an excellent prognosis [14]. Age and response to treatment are also prognostic factors [15]. Women fare better than men and this is independent of stage and age.

Laboratory Parameters

Several serum factors have been identified as prognostic indicators in early-stage CLL. Among patients with early-stage CLL (Binet stage A, Rai stage 0–2) considered to have "smoldering disease" (blood hemoglobin greater than 13.0 g/dL, a low absolute lymphocyte count (<30,000/μL), a lymphocyte doubling time greater than 12 months, and a nondiffuse pattern of lymphoid bone marrow infiltration [14]), serum thymidine kinase (TK) level >7.0 U/L identifies a group with significantly shorter progression-free survival (PFS) compared to those with lower TK levels [16]. Elevated serum β2-microglobulin level is also an adverse prognostic feature [17]. Serum soluble CD23 segregates Binet stage B disease into more or less aggressive forms [18]. High serum LDH levels indicate a poor prognosis [19]. These parameters appear to be surrogate markers of disease burden or cellular turnover.

Table 9.3 Staging of CLL

Rai stage [9]	Modified Rai stage [11]	Description	Binet stage [10]	Description	Median survival
0	Low risk	Lymphocytosis	A	Two or fewer lymphoid bearing areas	>10 Years
1	Intermediate risk	Lymphocytosis and lymphadenopathy	B	Three or more lymphoid bearing areas	5–7 Years
2	Intermediate risk	Lymphocytosis and splenomegaly with/without lymphadenopathy			
3	High risk	Lymphocytosis and anemia (hemoglobin <11 g/dL)	C	Anemia (hemoglobin <10 g/dL) or thrombocytopenia (platelets 100×10^6/dL)	2–3 Years
4	High risk	Lymphocytosis and thrombocytopenia (platelets $<100 \times 10^6$/dL)			

A series of new prognostic markers have been identified. Testing for these is not needed for the diagnosis of CLL but may help clinicians to have an informed discussion with patients regarding prognosis. As most patients with CLL are diagnosed with early-stage disease, these data aid in predicting the likelihood of disease progression. Also, some (although not all) of the prognostic markers are correlated with response to therapy.

Genetic Studies

Using conventional chromosome banding techniques, cytogenetic abnormalities can be detected in 40–50% of cases of CLL [20]. This technique is hampered by the low mitotic activity of CLL cells; B-cell mitogens may be used to enhance this activity. In addition, metaphases obtained for karyotyping after mitogen stimulation may arise from normal T cells in the sample [21].

Fluorescence in situ hybridization (FISH) using genomic DNA probes has greatly enhanced the ability to detect molecular abnormalities in malignant cells. This technique can detect aberrations in interphase cells. FISH has demonstrated that molecular abnormalities occur in up to 80% of cases of CLL [22].

13q deletion is the most common genetic aberration found in CLL by FISH (55%) followed by 11q deletion (18%), 12q trisomy (16%), and 17p deletion (7%) [22]. Prior to the use of FISH, trisomy 12 was the most frequently detected chromosomal abnormality in CLL by conventional cytogenetic methods. Structural abnormalities of 13q were often missed by Giemsa banding, presumably because of the small size of the deletion. The prognosis of CLL varies with the chromosomal abnormality. When divided into five prognostic categories, 17p deletion, 11q deletion, 12q trisomy, normal karyotype, and 13q deletion (as sole abnormality), the survival times were 32, 79, 114, 111, and 133 months [22], respectively. Patients with 17p or 11q deletion [23] had more advanced disease with frequent splenomegaly, mediastinal and abdominal lymphadenopathy, as well as more extensive peripheral lymphadenopathy. As part of clonal evolution, patients with CLL can acquire additional mutations. Therefore, at the time of relapse, it is advisable to repeat FISH prior to selection of therapy.

The search for tumor-suppressor genes in the commonly deleted region of 13q14 led to the discovery that two microRNA genes, mir-15a and mir-16, are located in this region [24] and in majority of patients with CLL (approximately 68%) these microRNAs are deleted or downregulated. These two microRNAs are inversely linked to the expression of the antiapoptotic Bcl-2 protein at a posttranslational level [25]. Thus loss of these two microRNAs can be linked to apoptosis resistance in CLL cells through upregulation of Bcl-2. Additional targets of mir-15 and -16 include proteins related to cell cycle progression [26]. The 11q22-q23 deletion is associated with loss of the ataxia telangiectasia mutated (ATM) [27], a gene that is responsible for repair of DNA double-stranded breaks (DNA DSB), activation of cell cycle check points, and inducing of apoptosis in response to DNA DSB. ATM also functions directly in the repair of chromosomal DNA DSBs by maintaining DNA ends in repair complexes generated during VDJ gene rearrangement in the lymphocyte receptor assembly [28]. This explains the occurrence of lymphoid malignancies in patients with ataxia telangiectasia as chromosomal instability in the lymphoid population arises at a time when lymphoid cell receptor diversity is established. The residual ATM allele is mutated in 36% of CLLs with an 11q deletion [29]. Deletion of 17p13 in CLL always includes loss of tumor suppressor TP53 [30]. Deletion of the TP53 gene is associated with poor overall survival (OS) and chemoresistance [30, 31]. In CLL cases with monoallelic loss of TP53, the other allele is mutated in the vast majority [32]. TP53 mutation even in the absence of 17p13 abnormality is associated with poor OS in CLL [33]. The frequency of TP53 mutations is higher among patients with relapsed/refractory disease.

Somatic Hypermutation of Immunoglobulin Heavy-Chain Variable Gene

Recombination of variable (V), diversity (D), and joining (J) genes and insertion of nontemplated nucleotides at the V–D and D–J junction occur in the pregerminal phase of B-cell development. In addition to the diversity brought about by such VDJ recombination, somatic hypermutations are introduced in the V(D)J rearrangement in normal B cells in the germinal center to increase the B-cell repertoire. Assessment for somatic hypermutation in the immunoglobulin heavy-chain variable gene (IgV_H) defines two "subsets" of CLL. Approximately 50% of CLL cases have somatic hypermutation of the IgV_H gene and thus appear to arise from post-germinal B cells while the subset of CLL lacking IgV_H gene hypermutation appears to arise from naive B cells [34]. The mutation status of CLL cells appears fixed and mutational status is not gained or lost during the course of disease. The prognosis of patients with unmutated IgV_H gene is worse than that of patients with IgV_H mutations; the unmutated population is more likely to have advanced-stage and progressive disease [34, 35], as well as markedly shorter PFS and OS after chemoimmunotherapy. The prognostic value of mutation status may be changing in the era of targeted inhibitors.

Stereotyped B-Cell Receptors

Study of VDJ rearrangement of immunoglobulin gene in CLL indicates that certain gene segments are overrepresented across different patients, indicating a stereotyped use

of these gene segments (an event occurring more frequently than by chance). A higher proportion of unmutated CLL cases carry stereotyped VDJ rearrangements resulting in similar complementarity-determining regions (CDRs) [34]. More than 20% of CLL cases can carry stereotyped BCRs which suggests exposure to similar antigens which are related to the pathogenesis [36–40]. Use of stereotyped IgV_H genes also affects prognosis. The use of IgV_H 3–21 gene is associated with an aggressive clinical course independent of the IgV_H mutation status [41].

ZAP-70 and CD38 Expression

An attempt was made to identify surrogate markers for IgV_H mutation status. A gene expression analysis identified ZAP-70, a tyrosine kinase protein normally expressed in T and NK cells, to be differentially expressed between mutated and unmutated CLL cases [42]. Immunophenotypic analysis of CLL cases with known mutation status identified higher CD38 expression in cases with unmutated IgV_H [35]. Thus CLL cell expression of ZAP-70 [43–46] and CD38 [35] tends to correlate with unmutated IgV_H and predict poor prognosis. However, their correlation with unmutated IgV_H is not absolute [47, 48], and CD38 and IgV_H mutation status can be independent prognostic factors. Moreover, unlike IgV_H mutation status, CD38 expression can change with time.

Factors That Identify Patients More Likely to Need Treatment

The CLL Research Consortium evaluated the relative value of ZAP-70, CD38 expression, and IgV_H mutation status for predicting time to treatment in patients with newly diagnosed CLL [49]. Based on the analysis of these three parameters, patients can be divided into three risk groups: low, ZAP-70 negative and IgV_H mutated; intermediate, ZAP-70 negative and IgV_H unmutated; and high, ZAP-70 positive irrespective of mutation status. Though ZAP-70 expression can predict for need for treatment, there are concerns about standardization of the procedure to detect ZAP-70 including whether flow cytometry or immunohistochemistry should be used and appropriate gating methods to detect ZAP-70 by flow cytometry. Information about CD38 expression did not appear to add any further prognostic information.

Combining FISH and IgV_H mutation status, the group in Ulm divided patients with early-stage CLL into three risk groups [50]: high risk, del (17p13) irrespective of IgV_H mutation status; intermediate risk, del (11q22) and/or unmutated IgV_H; and low risk, IgV_H mutated in the absence of del (17p13) or del (11q22).

Factors That Impact Response to Therapy

Deletion 17p13 and TP53 mutations are associated with resistance to treatment with nucleoside analogs or alkylating agents and their combinations. IgV_H mutation status does not predict for response, but responses (to chemoimmunotherapy) in patients with IgV_H mutations last longer [51, 52]. Ibrutinib does have activity in CLL with del(17p) [53].

Factors That Impact Overall Survival

Based on clinical and laboratory characteristics of 1674 patients with previously untreated CLL presenting to MDACC, Wierda et al. [54] developed a nomogram comprised of easily available parameters that include age, β2-microglobulin, absolute lymphocyte count, sex, Rai stage, and number of involved lymph node groups. This nomogram predicts for survival probability at 5 and 10 years.

Indications for Treatment

Outside the auspices of a clinical trial, patients with asymptomatic early-stage CLL (Rai stage = 0, Binet stage = A) should be observed until there is evidence of disease progression [2]. Patients with advanced disease (modified Rai stage intermediate or high or Binet B and C) may benefit from therapy. In addition to advanced stage, evidence of active disease should be present to initiate therapy. Such indicators of active disease include (1) marrow failure indicated by cytopenia, (2) splenomegaly (>6 cm below the costal margin or symptomatic splenomegaly), (3) massive (>10 cm) or symptomatic lymphadenopathy, (4) lymphocyte doubling time of <6 months, (5) autoimmune hemolytic anemia or thrombocytopenia poorly responsive to corticosteroids, and (6) a minimum of one disease-related symptom: (a) unintentional weight loss of ≥10% within the previous 6 months, (b) significant fatigue, (c) fevers for ≥2 weeks without any evidence of infection, and (d) night sweats for more than 1 month.

Treatment of CLL

Use of single alkylating agents such as chlorambucil for frontline therapy of CLL is now of historical interest though such an approach may have a role in the treatment of elderly patients with CLL and/or patients with significant comorbidities. See Table 9.4 for commonly used regimens in CLL. Purine analogs have shown single-agent activity and combinations built around the use of purine analogs have become the standard of care for patients with CLL.

Table 9.4 Commonly used chemoimmunotherapy regimens in CLL

Regimen	Schedule	ORR/CR	MRD negative	Remission duration
Fludarabine + rituximab [63]	F 25 mg/m² day 1–5	90/47	N/A	70% at 2 years
	R 375 mg/m² day 1 for 6 cycles followed by R 375 mg/m² weekly × 4			
Fludarabine + cyclophosphamide + rituximab (FCR) [65, 66]	**F** 25 mg/m² days 1–3	95/70	78% of CRs	68% at 5 years
	C 250 mg/m² days 1–3			
	R 500 mg/m² day 1			
	For 6 cycles			
Pentostatin + cyclophosphamide + rituximab (PCR) [69]	**P** 2 mg/m² day 1	91/41	73% of CRs	48% at 26 months
	C 600 mg/m² day 1			
	R 375 mg/m² day 1			
	For 6 cycles			
Bendamustine + rituximab (BR) [71]	**B** 70 mg/m² day 1–2	88/23	29.2% of CRs in bone marrow; 57.8% in peripheral blood only	
	R 375 mg/m² day 1			
	For 6 cycles			

Fludarabine

Fludarabine is the most extensively tested nucleoside analog in CLL. Cellular pharmacology suggests that intracellular accumulation of fludarabine triphosphate is dependent on both drug concentration and duration of exposure [55]. Thus a low-dose repeated dosing schedule was adopted for treatment of CLL.

Pentostatin or Deoxycoformycin

Deoxycoformycin is an inhibitor of adenosine deaminase (ADA), and based on lymphopenias observed in patients with ADA deficiency, pentostatin has been tested as single agent in CLL, both in frontline and salvage settings, yielding modest activity [56, 57]. Pentostatin is perceived to be less myelosuppressive than fludarabine and that led to it being investigated as part of chemoimmunotherapy regimens that will be discussed later.

Bendamustine

Bendamustine is a potent alkylating agent with a low rate of cross-resistance with other alkylating agents. In a phase III randomized, open-label trial comparing bendamustine to chlorambucil in previously untreated patients with CLL, the ORR (68% vs. 31%), CR rate (31% vs. 2%), and PFS (median, 21.8 months vs. 8.0 months) were better in the bendamustine-treated patients [58].

Combination of Purine Analogs with Other Chemotherapeutic Agents

Alkylating agents have been tested in combination with purine analogs. The rationale for such combinations is based on the fact that alkylating agents induce base excision, nucleotide excision, and mismatch repair. This involves removal of damaged nucleotides followed by resynthesis. Exposure to alkylating agents results in more CLL cells requiring DNA resynthesis. At this resynthesis step purine analogs are incorporated in DNA strand repair patch, stop elongation of DNA strands, and induce apoptosis [59].

Fludarabine/Pentostatin and Cyclophosphamide

In a cohort of 128 patients with CLL that included untreated and previously treated (including fludarabine refractory) patients, the combination of fludarabine (30 mg/m² intravenously daily for 3 days) and cyclophosphamide (FC) showed an ORR of ≥80% [60]. The cyclophosphamide dose was decreased from 500 mg/m²/day for 3 days to 300 mg/m²/day for 3 days because of myelosuppression in the early part of the study. The response to FC was higher compared to historical responses to single-agent fludarabine among patients undergoing salvage therapy, with a 38% response rate among patients refractory to fludarabine. While the CR rate (35%) was comparable to fludarabine alone among previously untreated patients, minimal residual disease elimination at the end of therapy was achieved at a higher rate compared to fludarabine alone. The German CLL Study Group (GCLLSG) reported similar activity with FC [61]. Myelosuppression leading to infections was the most common side effect of therapy in both studies.

Chemoimmunotherapy

Fludarabine and Rituximab

The combination of fludarabine and rituximab has been shown to have a synergistic effect against lymphoma cell lines [62]. This led to clinical investigations involving this

combination. CALBG 9712 study compared concomitant fludarabine and rituximab with sequential regimen of fludarabine followed by rituximab in patients with previously untreated CLL [63]. The ORR and CR rate was higher in the concomitant arm. A retrospective comparison of 104 patients treated with fludarabine and rituximab on CALB 9712 with 171 patients treated with fludarabine alone in CALBG 9011 study indicated better OS and PFS in patients enrolled on CALBG 9712 [64].

Fludarabine, Cyclophosphamide, and Rituximab

Based on single-agent activity of rituximab in other studies and its synergism with fludarabine, the group at MD Anderson Cancer Center pioneered the combination of rituximab with the most effective chemotherapy combination of fludarabine and cyclophosphamide (FCR) [65]. In their initial report, they noted an ORR of 95% (CR = 70%, nodular PR = 10%, PR = 15%) with the combination regimen of fludarabine, cyclophosphamide, and rituximab (FCR). The CR rate with this regimen was significantly higher than that reported with FC. Moreover, 78% of the patients achieving CR also achieved MRD-negative status as assessed by flow cytometry [defined as CD5- and CD19-coexpressing cells of less than 1%, with normalization of the kappa:lambda ratio (<3:1 in patients with monotypic kappa and >1:3 in patients with monotypic lambda)]. Cytopenias precluded completion of the planned six cycles of treatment in 13% of patients. Neutropenia (≥ grade 3) was encountered in 52% of courses administered to all patients, but only 2.6% of these courses were associated with serious infectious episodes.

Long-term follow-up (median follow-up 6 years) results of this chemoimmunotherapy regimen administered to 300 patients showed a 6-year overall survival of 77% and progression-free survival of 51% with a median time to progression of 80 months [66].

Though all response parameters are superior with the FCR regimen compared to historical data with the FC regimen, a demonstrated survival benefit in a randomized comparison was lacking. The German CLL Study Group performed a multicenter randomized phase III trial [67] involving 817 patients; they reported a better overall survival in patients with previously untreated CLL with the FCR regimen compared to the FC regimen [84.1% in the FCR arm versus 79.0% in the FC arm ($p = 0.01$)]. This improvement in survival was seen in patients with Binet stage A and B CLL. Though cytopenias were more common in the FCR arm, no increased serious infectious episodes were seen (compared to the rate with FC). A multivariate analysis confirmed the beneficial effect of FCR regimen on OS and PFS.

In patients with previously treated CLL, the MD Anderson group reported that the FCR regimen produced a 73% ORR and 25% CR rate [68].

Pentostatin, Cyclophosphamide, and Rituximab

Clinical activity of the combination of pentostatin and cyclophosphamide also encouraged the chemoimmunotherapy regimen of pentostatin, cyclophosphamide, and rituximab (PCR) (see discussion to come). In an initial report of 64 patients, the ORR was 91% and the CR rate was 41% with 23% of patients in CR achieving a MRD-negative status (≤1% positive $CD5^+/CD19^+$ cells) [69]. A total of five patients required transfusions; grade 3/4 cytopenias were encountered in 14.5% of cycles with grade 3/4 infectious complication in only 2% of the cycles.

The initial expectation with the PCR regimen was that the infectious complications would be less than those seen with FCR regimen. However, a randomized community-based trial in previously untreated or minimally pretreated patients comparing the PCR regimen to FCR reported a better CR rate with FCR with a comparable overall response rate, as well as a comparable rate of cytopenias and infectious complications [70].

Bendamustine Rituximab

In a phase II trial of the GCLLSG, bendamustine was combined with rituximab in previously untreated patients with chronic lymphocytic leukemia [71]. The overall response rate was 88% with complete response rate of 23% and partial response rate of 64.9%. At a median follow-up of 27 months, median event-free survival was 33.9 months. The rate of grade 3 or 4 severe infection was 7.7%. Response to therapy was associated with cytogenetics. While 90% of patients with del(11q) and 94.7% of patients with trisomy 12 responded, only 37.5% of patients with del(17p) responded.

The CLL 10 study of the GCLLSG was a phase III, open-label, randomized study comparing bendamustine and rituximab (BR) to FCR [72]. At a median follow-up of 37.1 months, the median progression-free survival was 41.7 months with bendamustine and rituxan and 55.2 months with FCR. However, severe neutropenia and infection were more frequent in the FCR group. The incidence of infectious complications in the FCR group was more pronounced in patients older than 65 years. However, there was a benefit seen in patients with CLL with del(11q) with FCR over bendamustine and rituximab. Of note, patients with del(17p) were not included in this study. Therefore, FCR remains a standard of care in fit patients. However, bendamustine and rituximab are associated with less infectious complications.

Antibodies

CD20 Antibodies

Rituximab, ofatumumab, and obinutuzumab are three anti-CD20 antibodies approved for the treatment of CLL. Ublituximab is being developed as a novel anti-CD20 antibody. CD20 is an antigen expressed on the surface of CLL cells (dim expression by flow cytometry) and is tightly bound to the cell surface. The mechanisms of action of anti-CD20 antibodies against CLL cells include antibody-dependent cellular cytotoxicity [73] (ADCC) and complement-mediated cytotoxicity [74]. In addition, exposure of CLL cells to anti-CD20 antibody has been shown to reduce levels of antiapoptotic proteins including XIAP and Mcl-1 and to induce caspase activation and PARP cleavage [75].

Rituximab

Rituximab is a chimeric monoclonal antibody approved for the treatment of low-grade B-cell lymphomas. The pivotal trial in patients with relapsed low-grade B-cell lymphomas using 375 mg/m^2 weekly for 4 weeks showed responses in 48% of patients, but the response rate among patients with SLL (tissue equivalent of CLL) was 12% [76]. This was attributed to the fact that expression of CD20 was low on SLL/CLL cells compared to cells of follicular lymphomas. O'Brien et al. [77] conducted a dose escalation study of rituximab (375 mg/m^2 dose 1 and dose 2–4 at an escalated rituximab dose). For each patient, the dose of rituximab was kept constant and escalation range was 500–2250 mg/m^2. The ORR among patients with CLL was 36%, all responses being PR.

While Fcgamma RIIA [78] and RIIIA [79] gene polymorphism has been linked to responses to rituximab in patients with non-Hodgkin's lymphoma, no such association has been convincingly established in CLL [80].

Ofatumumab

Ofatumumab is a CD20 monoclonal antibody that binds to a unique epitope that is distinct from the epitope recognized by rituximab. Ofatumumab produces more complement-dependent cytotoxicity (CDC) than is seen with rituximab. As a single agent in patients with relapsed/refractory CLL, the overall response rate was 45% with a median PFS of 5 months [81]. Based on these results, ofatumumab was approved for patients with CLL refractory to fludarabine and alemtuzumab. Ofatumumab has been combined with chlorambucil as first-line therapy (Complement-1) trial [82]. The chlorambucil and ofatumumab combination significantly improved ORR compared to that seen with chlorambucil monotherapy (82% vs. 69%, $p < 0.001$). The combination also improved PFS (median 22.4 months vs. 13.1 months, $p < 0.001$). While ofatumumab is generally well tolerated, the most common side effects are infusion reactions and neutropenia. Based on these results, chlorambucil and ofatumumab were approved for first-line treatment of patients with CLL for whom fludarabine-based therapy is considered inappropriate.

Obinutuzumab

Obinutuzumab is a CD20 monoclonal antibody with a glycoengineered Fc portion leading to enhanced antibody-dependent cellular cytotoxicity (ADCC), increased direct cell death, and lower complement-dependent cytotoxicity (CDC) compared to that seen with rituximab [83]. The CLL11 trial randomized 781 previously untreated patients with CLL with comorbidities to receive chlorambucil monotherapy, chlorambucil with rituxan, or chlorambucil with obinutuzumab. The combination of chlorambucil and obinutuzumab produced an improved PFS compared with that seen with chlorambucil alone (26.7 vs. 11.1 months, $p < 0.001$) [84]. The combination of chlorambucil and obinutuzumab also showed an improved PFS when compared with the PFS noted with the chlorambucil and rituximab combination (median 26.7 vs. 16.3 months, $p < 0.001$). Chlorambucil and obinutuzumab produced a higher ORR as compared to that seen with chlorambucil and rituximab (78.4% vs. 65.1%, $p < 0.001$). These results led to FDA approval of the combination of chlorambucil and obinutuzumab for patients with previously untreated CLL not suitable for more aggressive chemoimmunotherapy. In a recent update, chlorambucil and obinutuzumab showed an improved PFS compared with chlorambucil and rituximab (29.2 months vs. 15.4 months, $p < 0.001$) [85]. However, no overall survival difference was noted between the two antibody arms.

Ublituximab

Ublituximab (TG-1101) is a chimeric IgG1 monoclonal antibody that targets a unique epitope on CD20. In a phase I trial, ublituximab demonstrated a 67% ORR in patients with relapsed/refractory CLL [86]. The combination of ublituximab and ibrutinib is being investigated. In an initial report of 20 patients with high-risk CLL, the overall response rate was 95% [87]. Final results of this trial are yet not reported.

CD52 Antibody

Alemtuzumab, originally known as Campath 1G, is a human immunoglobulin G1 (IgG1) anti-CD52 monoclonal antibody (MAb) that binds to nearly all B- and T-cell lymphomas and

leukemias. Early phase II study with an administration schedule of a 30-mg 2-h intravenous (IV) infusion thrice weekly for a maximum period of 12 weeks produced an ORR of 42% [88]. Most disease elimination was seen in blood, bone marrow, and spleen while lymph node response was less. Keating et al. [89] reported on an international study involving 93 patients with fludarabine-refractory CLL using alemtuzumab 30 mg IV three times a week for 12 weeks. In the first week, the initial dose was 3 mg, which was increased to 10 mg, and then to 30 mg as soon as infusion-related reactions were tolerated. Infection prophylaxis with trimethoprim/sulfamethoxazole and famciclovir was mandatory. The intent-to-treat analysis showed an ORR of 33% (CR = 2%, PR = 31%). Though there was reduction in lymphadenopathy and other organomegalies, response in lymph nodes >2 cm was modest. In this pivotal trial, 25 patients had grade 3/4 infectious complications. Viral reactivation (cytomegalovirus = seven patients and herpes simplex virus = six patients) was seen in 13 patients. Infectious complications were more frequent in nonresponders to alemtuzumab than in responders.

Alemtuzumab is no longer available commercially but can be provided through the company through a distribution program.

Targeting Antiapoptotic Proteins

Antiapoptotic members of the Bcl-2 family of proteins can render CLL cells resistant to chemotherapeutic agents; increased expression of Bcl-2 family members is frequently seen in primary CLL cells [90]. Drugs targeting antiapoptotic members of the Bcl-2 family of proteins can induce apoptosis in CLL cells [91] and have synergistic/additive effect with chemotherapeutic agents.

In addition to antisense oligonucleotides, small-molecule inhibitors of Bcl-2 family members have been developed. These agents mimic the BH3 domain of pro-apoptotic Bcl-2 family proteins and work by releasing BH3 only pro-apoptotic members (BAX and BAK) from sequestration by the antiapoptotic members.

Venetoclax

Antiapoptotic proteins such as BCL-2 are expressed at high levels which make the cells resistance to senescence and death. Venetoclax is an oral BCL-2 inhibitor. In the phase I trial, 56 patients with relapsed chronic lymphocytic leukemia were treated in the dose-escalation cohort [92]. Clinical tumor lysis was diagnosed in three patients in that cohort with one death. Adjustments were made in the dose-escalation schema, increased tumor lysis prophylaxis was employed, and initial hospitalization was required, and the subsequent 60 patients tolerated the drug well. There was a 79% overall response rate with 20% complete responses. The 15-month progression-free survival was 69% in the 400 mg cohort. Other side effects were mild diarrhea (52%), upper respiratory tract infection (48%), nausea (47%), and grade 3 or 4 neutropenia (41%). In a phase II trial, 107 patients with relapsed or refractory CLL with 17p deletion were treated with venetoclax [93]. At a median follow-up of 12 months, the overall response rate was 85%. Most common grade 3–4 adverse events were neutropenia (40%), infection (20%), anemia (18%), and thrombocytopenia (15%). Serious adverse events included pyrexia, autoimmune hemolytic anemia (7%), pneumonia (6%), and febrile neutropenia (5%). These results led to the recent FDA approval of venetoclax in relapsed or refractory patients with CLL and del 17p.

Inhibitors of the B-Cell Receptor

B-cell receptor activation is known to play a crucial role in the pathogenesis of CLL. There are many potential targets for inhibition of the B-cell receptor pathway. Kinases for which targeting agents are commercially available include BTK and PI3K [94].

"Redistribution Lymphocytosis"

As a class effect, all inhibitors of the B-cell receptor appear to cause "redistribution lymphocytosis." During the first few weeks of therapy, these agents can cause transient lymphocytosis due to redistribution of CLL cells from the tissue to peripheral blood [95]. Normally, CLL cells circulate in the peripheral blood where they are attracted to tissue stromal cells by a chemokine gradient. The CXCR4-CXCL12 axis is the predominant one for marrow homing. Inhibition of these homing mechanisms by ibrutinib, a BTK inhibitor, leads to the exit of tissue cells into the blood, resulting in an increased lymphocytosis. Lymphocytosis occurs concomitantly with reduction in lymph node size [95]. The transient lymphocytosis should not be confused with disease progression and should not lead to discontinuation of the drug (Fig. 9.1).

Since this class of drugs is known to cause lymphocytosis, many patients do not meet response criteria defined in the 2008 International Workshop on Chronic lymphocytic leukemia (IWCLL) guidelines [2] despite clear and substantial clinical benefit. Thus, the IWCLL guidelines have been adjusted to define an initial response category called partial response with lymphocytosis (PRL), thus ensuring that patients with a partial response who have persistent lymphocytosis are considered responders [96].

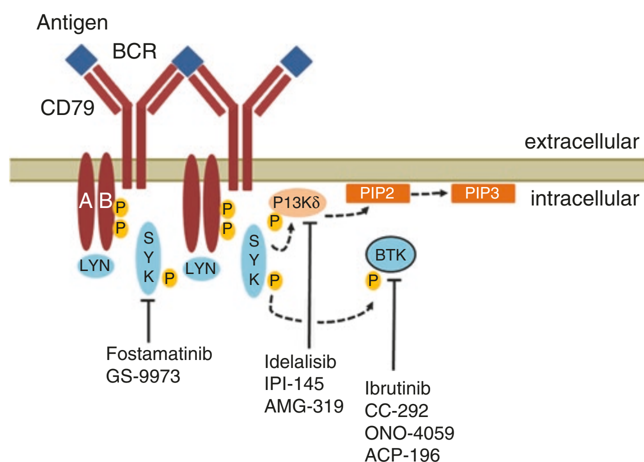

Fig. 9.1 BCR signaling and downstream pathways. The BCR consists of transmembrane receptors. Upon binding of antigen, BCR signaling induces LYN- and SYK-dependent phosphorylation of tyrosine motifs including PI3Kδ and BTK. Several small molecular inhibitors to this kinases are in development (adapted from Wiestner 2014 [94])

Bruton's Tyrosine Kinase (BTK)

BTK is a cytoplasmic tyrosine kinase which is essential to BCR signaling and couples BCR-induced calcium release to activation of the NK-κB pathway and cellular proliferation. X-linked agammaglobulinemia (or Bruton's agammaglobulinemia), which typically presents during childhood, is characterized by an absence of mature B cells and immunoglobulins and leads to recurrent bacterial infections. Loss-of-function mutations in BTK block B-cell maturation at the pre-B-cell stage [97].

Ibrutinib

Ibrutinib is the first BTK inhibitor studied in clinical trials; it inactivates BTK through the formation of an irreversible covalent bond with Cys-481 in the ATP-binding domain [98]. Ibrutinib is also known to inhibit other kinases including ITK (interleukin-2-inducible T-cell kinase), TEC, BMX, and EGFR, which may explain some of the toxicities seen with this agent. Ibrutinib is currently FDA approved for use as initial therapy as well as for the treatment of relapsed disease.

Byrd et al. reported the outcomes of 101 patients with relapsed/refractory CLL, who received ibrutinib [99, 100]. The median age was 64 years old (range, 37–82). Thirty-four percent of the patients had del(17p) and 78% had unmutated IGVH. The median number of prior regimens was four. The overall response rate was 90% with 7% with complete remission (CR). The estimated progression-free survival at 30 months in this heavily pretreated group was 69%. The median PFS in patients with del(17p) and del(11q) was 28 months and 38.7 months, respectively. While this median PFS is a significant improvement over prior experience with chemotherapy regimens in this high-risk group, there is clearly a shorter PFS in patients with del(17p). The most common toxicity was diarrhea which occurred in 55% of patients and was predominately grade 1–2. Notable toxicities >grade 3 included bleeding (8%) and atrial fibrillation (6%).

Ibrutinib was compared to ofatumumab in previously treated patients with CLL in a randomized trial, with results demonstrating improved progression-free and overall survival in the ibrutinib arm [101]. At 12 months, the overall survival was 90% in the ibrutinib arm versus 81% in the ofatumumab arm. The overall response rate was higher with ibrutinib at 43% versus 4% with ofatumumab.

Patients with CLL and a 17p deletion typically have aggressive disease and respond poorly to chemotherapy regimens [102]. A recent report described the outcome of 144 patients with CLL and a 17p deletion who had failed at least one therapy and received ibrutinib 420 mg daily until disease progression [53]. The median number of prior therapies was two with a range of 1 to 7 prior therapies. At a median follow-up of 13 months, the median PFS had not been reached. At 12 months, 79% were alive and progression free and 88% of the responders were progression free. Progressive disease was reported in 13% of patients; 7% of patients developed Richter's transformation and seven of those cases occurred within the first 24 weeks of therapy. The most frequent adverse events were diarrhea in 36% (grades 3–4 in 2%), fatigue in 30% (grades 3–4 in 1%), cough in 24% (grades 3–4 in 1%), and arthralgias in 22% (1% with grades 3–4). Another report described 51 patients with CLL and deletion 17p who were treated with ibrutinib [103]. At 24 weeks, 80% of patients had an objective response: 40% had a PR, 40% had a PRL, and the remaining 20% had stable disease. The adverse effects were similar to those seen in previous reports.

The frontline trial leading to approval of ibrutinib as initial therapy randomized 269 patients who were 65 years or older to receive either ibrutinib or chlorambucil [104]. With a median follow-up of 18.4 months, the median progression-free survival was not reached with ibrutinib versus 18.9 months with chlorambucil. The 24-month overall survival with ibrutinib was 98% versus 85% with chlorambucil.

Toxicity

The incidence of bleeding with ibrutinib was found to be increased. In the phase I/II studies, bruising was seen in 17% of patients: 2% had intracranial hemorrhage [105]. Subsequent trials with ibrutinib have excluded patients on warfarin therapy. In the phase III trial of ibrutinib versus ofatumumab, the bleeding rates were 44% with ibrutinib and

12% with ofatumumab but the rates of serious bleeding were low at 1% versus 2%, respectively [101]. An earlier analysis of the bleeding events with ibrutinib was attributed to effects on the collagen and von Willebrand-dependent platelet functions [106]. However, a more recent analysis attributes the bleeding to inhibition of collagen-dependent platelet aggregation by ibrutinib [107].

The incidence of atrial fibrillation is also increased with ibrutinib. In the phase III trial of ibrutinib versus ofatumumab, ten patients on the ibrutinib arm developed atrial fibrillation versus only one patient on the ofatumumab arm. This led to discontinuation of ibrutinib in one patient [101].

Finally, as with other BCR inhibitors, ibrutinib does cause lymphocytosis. This generally resolves after 6–9 months with continued treatment. Approximately 20% of patients have prolonged lymphocytosis (greater than 12 months) with ibrutinib therapy. Development of lymphocytosis does not appear to be detrimental to long-term clinical outcomes [108].

Ibrutinib Resistance

The outcome of heavily pretreated patients who fail ibrutinib is poor. A previous report described outcomes in 33 patients (26%) (of 127 patients) enrolled on clinical trials of ibrutinib at MD Anderson who discontinued the drug [109]. The majority of these patients had high-risk features including 94% with unmutated IgVH, 58% with 17p deletion by FISH, and 54% with complex karyotype. The reason for discontinuation of ibrutinib included disease transformation in 7%, progressive disease in 7%, and adverse events in 11%; 3% of patients underwent stem cell transplantation. Seventy-six percent of patients died after discontinuing ibrutinib, with a median overall survival after discontinuation of only 3 months. These patients who discontinued ibrutinib had aggressive disease and were heavily pretreated. Recent analysis of outcomes of patients with ibrutinib therapy demonstrated that this does not hold true for patients who receive ibrutinib earlier in their disease course [110]. Patients who were treatment naïve in the Resonate-2 trial and patients that had several lines of prior therapy prior to ibrutinib were compared. The median overall survival in patients post-ibrutinib therapy who had ibrutinib as either first- or second-line therapy was not reached. The median overall survival in patients who had ibrutinib as third-line (or beyond) therapy was only 7–9 months. Therefore, patients who receive ibrutinib in first or second line of therapy were less likely to progress and also experienced better post-ibrutinib survival.

Mutations in the BTK-binding site of ibrutinib have been described in patients who developed resistance while on ibrutinib therapy [111]. Mutations in the cysteine-to-serine in BTK at position 481 (C481S) were seen in five patients. The C481S mutation elicits BTK-independent activation after B-cell receptor activation [112]. Two patients had mutations in PLCγ2 (a downstream kinase from BTK), one at position 665 (R665W) and the other at L845F, leading to gain-of-function mutations that led to potential autonomous B-cell receptor activity. Proximal kinases of SYK and LYN would be critical for activation of mutant PLCG2 and targeting LYN and SYK may combat molecular resistance in cell line models as well as in primary CLL cells from ibrutinib-resistant patients. There has also been a report of clonal evolution leading to ibrutinib resistance. One patient acquired a new clonal mutation in SF3B1(K666 T) and two patients had clonal deletion in chromosome 8p [113]. Other proposed mechanisms for resistance to ibrutinib as well as the other BCR antagonists are reviewed elsewhere [114].

Clinical data are limited on how to proceed after a patient has disease progression on ibrutinib. These patients may be considered for stem cell transplantation, if they are eligible candidates for this intervention [115]. This group may benefit from development of other novel therapies. Because the current second-generation BTK inhibitors bind to the same binding site as ibrutinib, using them in this setting would likely not be productive. A phase I study of the dual-PI3K inhibitor IPI-145 included some patients previously treated with ibrutinib [116]. There was one PR and five patients had stable disease. For further details, refer to the section on IPI-145.

Based on ex vivo data supporting the use of selinexor in the setting of acquired resistance to ibrutinib, as well as in vitro synergy with ibrutinib in chronic lymphocytic leukemia [117], a clinical trial is investigating the combination of selinexor and ibrutinib in relapsed/refractory CLL [118].

Preliminary results from a phase II study of venetoclax in patients with CLL relapsed after ibrutinib or idelalisib are encouraging [119]. In this heavily pretreated group (median of 5 prior regimens) of 54 patients, 41 patients had had prior ibrutinib and 13 patients had prior idelalisib. In the group which had been pretreated with ibrutinib, 13% CR, 48% PR, and 13% SD at 24 weeks were seen. In the group who had been pretreated with idelalisib, the response rate was 50% PR and 25% SD at 24 weeks. At 36 weeks, the overall response rate was 61% in the ibrutinib-pretreated group and 50% in the idelalisib-pretreated group. The drug was well tolerated but serious adverse events were seen, 7% pneumonia and 7% neutropenia.

Second-Generation BTK Inhibitors

Other BTK inhibitors in earlier clinical development include GS-4059 (ONO-4059) [120, 121], acalabrutinib [122, 123], and BGB-3111 [124]. These inhibitors all covalently bind to Cys481 leading to irreversible inhibition. These second-generation inhibitors may have more selective binding to BTK and fewer off-target effects, such as diarrhea, bleeding, and atrial fibrillation.

GS-4059 (ONO-4059)

GS-4059 is a potent and selective BTK inhibitor with an IC50 in the sub-nmol/L range. In a phase I study, the oral inhibitor was given as monotherapy to patients with relapsed/refractory CLL [119]. Of the 16 patients evaluable, 38% of patients had a 17p deletion and 19% of patients had an 11q deletion. Twelve of 16 patients had an unmutated IgVH gene. Eight of 16 patients had a TP53 mutation. The median number of prior therapies was 3. This inhibitor was well tolerated; grade 3 toxicities included febrile neutropenia, as well as one grade 4 neutropenia. The best overall response was 70% as per IWCLL criteria. Two patients had a PR and five patients had a PRL, two had stable disease, and one progressed with Richter's transformation. The results in 60 patients with relapsed hematologic malignancies treated with GS-4059 were recently published [120]. Of 25 patients with relapsed CLL, 24 responded to and 21 patients remained on therapy. One patient had grade 3 bleeding but no diarrhea, cardiac events, or arthralgias were reported. There is an ongoing clinical trial evaluating GS-4057 in various combinations of other agents. There are five different arms comparing GS-4057 with idelalisib, GS-4057 with entospletinib, GS-4057 with idelalisib and obinutuzumab, GS-4057 with entospletinib and obinutuzumab, and just single agent GS-4057 [125]. There is an upcoming clinical trial comparing the combination of GS-4057 and idelalisib with or without obinutuzumab in patients with relapsed/refractory CLL [126].

ACP-196 (Acalabrutinib)

ACP-196 is a second-generation BTK inhibitor which binds covalently to Cys481 with improved selectivity and in vivo target coverage [121]. ACP-196 was able to inhibit 94% of BTK target occupancy after 7 days of dosing in patients with CLL. The results of a phase I–II multicenter study of ACP-196 in patients with relapsed CLL were recently published [122]. Sixty-one patients with relapsed CLL had received a median of three prior regimens and 31% had deletion 17p. No dose-limited toxicities were seen. At a median follow-up of 14.3 months, the overall response rate was 95%, including 85% with partial response and 10% with PRL. The remainder (5%) had stable disease. The response rate in the patients with deletion 17p was 100%. There is an ongoing registration trial randomizing patients with relapsed CLL and deletion 17p or 11q to ibrutinib or acalabrutinib [127]. The FDA registration phase III study for potential frontline approval randomizes patients to one of the three arms, acalabrutinib, acalabrutinib and obinutuzumab, or chlorambucil and obinutuzumab [126].

BGB-3111

BGB-3111 is another oral BTK inhibitor. Ibrutinib antagonizes rituximab-induced antigen-dependent cell-mediated cytotoxicity (ADCC) by inhibiting ITK kinase activity [128]. In murine models BGB-3111 resulted in a tenfold weaker inhibition of rituximab-induced ADCC and was threefold more potent than ibrutinib in target organs [129]. The results of a phase I study of BGB-3111 in patients with B-cell lymphoid malignancies were recently reported [130]. Of 25 patients enrolled, 8 patients had CLL. There were other patients including mantle cell lymphoma, Waldenstrom's, DLBCL, follicular lymphoma, marginal zone lymphoma, and hairy cell leukemia. There were no serious adverse events leading to drug discontinuation or adverse-related disease reported. Of 21 adverse events grade 3 or greater, only 3 were felt to potentially be related to the drug which were neutropenia. There were no grade 3 or 4 bleeding events or cases of atrial fibrillation. Of the CLL patients, two had stable disease and six had partial response. The results of 45 patients with CLL treated with BGB-3111 were recently presented [131]. The drug was well tolerated with 69% of patients without any AE greater than grade 1 within the first 12 weeks of therapy. The most frequent AEs were petechiae/bruising, upper respiratory infection, diarrhea, fatigue, and cough. Three serious adverse events were reported which possibly could have been related to the drug which were grade 2 cardiac failure, grade 2 pleural effusion, and grade 3 purpura. At a median follow-up of 7.5 months, the response rate was 90% with PR in 79% of patients and PR-L in 10%, and stable disease in 7%. These results are encouraging and further studies are planned.

PI3K Inhibitor

The PI3K pathway is a key component of survival in a variety of cancers including CLL. There are three classes of PI3K isoforms. Class I isoforms are made up of two subsets: 1A, which includes p110α, p110β, and p110δ bound by regulatory domains, and 1B, which is composed of p110-γ coupled with p101 [132]. The p110δ is abundantly expressed in CLL as the delta isoform is the most important isoform in hematologic cells. Idelalisib is the first PI3K inhibitor in combination with rituximab approved by the FDA in patients with relapsed CLL. PI3K inhibitors in clinical development include duvelisib, and TGR-1202.

Idelalisib

Idelalisib is a selective oral reversible inhibitor of the p110δ isoform of PI3Kδ. In the phase I study of idelalisib, 72% of patients had an objective response with a median

progression-free survival of 16 months [133]. Overall, the patients on this trial had less functional reserve than the patients on ibrutinib trials, with decreased renal function, therapy-induced myelosuppression, or major coexisting illness. Furthermore, at the optimal doses, progression-free survival was 32 months. Median overall survival was not reached, with a 36-month overall survival of 75%. The most common >grade 3 adverse events were pneumonia in 20%, neutropenic fever in 11%, and diarrhea in 6% of patients. At the second interim analysis, the phase III randomized clinical trial of idelalisib with rituximab versus placebo with rituximab reported that the addition of idelalisib led to an overall response rate of 77% versus 15% with rituximab plus placebo [133]. Furthermore, in the idelalisib and rituximab arm, there was a 12-month progression-free survival of 66% [134, 135]. Serious adverse events occurred in 40% of patients in the idelalisib arm. The most common adverse events were pneumonia, pyrexia, and febrile neutropenia. Grade 3 or higher transaminitis occurred in 5% of patients in the idelalisib arm with onset at 8–16 weeks. The study drug was withheld and four of six patients were successfully rechallenged. Furthermore, in the idelalisib arm, gastrointestinal and skin toxicities led to discontinuation of the drug in six patients. Because PI3kδ influences clonal expansion and differentiation of suppressor T cells, diarrhea and colitis may be an expected autoimmune toxicity. A phase II trial evaluated the combination of idelalisib and rituximab in previously untreated patients with CLL/SLL who were 65 years or older [136]. This combination produced a high ORR of 97%. However, the adverse side effect profile was higher in this previously untreated cohort compared to that seen in relapsed/refractory patients treated with idelalisib The incidence of transaminitis was 67% with 23% of patients having grade 3 or higher toxicity. Diarrhea or colitis was reported in 64% of patients; 42% was grade 3 or higher. On the colonoscopic biopsies, T-cell infiltration was present in the patients with colitis. The authors noted that T-cell levels are typically normal in previously untreated patients with CLL but are quite low in patients with relapsed/refractory disease. This is one possible reason for the increased toxicity noted in the previously untreated group compared with the relapsed/refractory group.

These findings were confirmed in another phase II trial evaluating idelalisib in the frontline setting [137]. This trial enrolled 24 patients with newly diagnosed CLL who were treated with 2 months of idelalisib followed by 6 months of the combination of idelalisib and ofatumumab. Hepatotoxicity was reported in 79% of patients with 54% being grade 3 or higher. The median time to development of transaminitis was 28 days which was prior to the administration of ofatumumab. The transaminitis did resolve with holding the drug and, in some cases, addition of immunosuppressants. This study showed that the toxicity was increased in the younger frontline patients, presumably because they have better functioning immune systems. Clinical trials with idelalisib in the frontline setting have been discontinued because of a higher rate of infections and death than was seen in the control arm.

IPI-145 (Duvelisib)

IPI-145 is another inhibitor of PI3K. It inhibits both the p110δ and the p110γ isoforms. Duvelisib antagonizes BCR cross-linking activated pro-survival signals in primary CLL cells [138] and causes direct killing of primary CLL cells in a dose-dependent fashion while it spares normal B cells. Furthermore, based on ex vivo models, duvelisib could possibly overcome ibrutinib resistance resulting from the BTK C481S mutation [116].

In a phase I/II study of monotherapy with IPI-145, 54 patients with relapsed/refractory CLL were enrolled [139]. The patients were heavily pretreated with 82% having received more than three prior lines of therapy. The median time from prior therapy was 3.5 months. Cytogenetics were poor risk with 49% having TP53 mutations or a 17p deletion and 89% having an unmutated IgVH. The expansion cohort enrolled patients at either 25 or 75 mg twice daily. The best overall response rate was 55% in 49 evaluable patients including 1 CR and 26 partial responses (PR). There were 21 patients with stable disease (in this study PRL was counted as stable disease) and 1 patient with progressive disease. The overall response rate was independent of dose or the presence of TP53/17p deletion. There was early resolution in the lymphocytosis. Overall, the drug was well tolerated with transient cytopenias, with 31% neutropenia, 11% thrombocytopenia, 15% febrile neutropenia, and 11% pneumonia. Treatment was discontinued in 31% of patients due to adverse events, and in another 24% of patients because of disease progression. DUO is a phase III study in relapsed/refractory CLL randomizing 300 patients to either duvelisib 25 mg twice daily or ofatumumab for up to 18 cycles [140].

In the phase I study a cohort of patients with CLL resistant to ibrutinib were treated with duvelisib [116]. This included six patients with relapsed/refractory CLL and six patients with aggressive B-cell NHL (aNHL, including two with DBLCL and four with Richter's transformation). Two patients received duvelisib at 25 mg twice daily and ten patients received duvelisib at 75 mg twice daily. All patients had received more than three prior therapies. The median time from prior therapy to duvelisib was 0.3 months and 67% of patients received it within 2 weeks of ibrutinib. The patients with CLL had received a median of four cycles of duvelisib. The best response in patients with relapsed/refractory CLL

was one PR; five patients had stable disease. Of these six patients, two patients remained on the drug for 8 and 9 months and four patients discontinued the drug due to disease progression or physician decision.

TGR-1202

TGR-1202 is a second-generation PI3Kδ inhibitor. In a phase I study, patients with relapsed/refractory hematologic malignancies were administered monotherapy with TGR-1202 orally once daily following a 3 + 3 dose escalation design [141]. Preliminary results from this phase I study were notable for PR in four of the six patients with CLL treated at doses above 800 mg daily. The nodal reduction occurred rapidly and was accompanied by lymphocytosis. Subsequent reporting revealed that of nine evaluable patients with CLL, eight (89%) achieved a PR in the nodes with a median nodal reduction of 71%, of whom five achieved a PR [142]. Notably, in comparison to other PI3kδ inhibitors, there were no cases of hepatotoxicity or colitis observed. Rates of infection and pneumonia were low at 12% and 6%, respectively, with no cases of febrile neutropenia.

Based on the encouraging phase I data, the combination of TGR-1202 and ublituximab (a glycoengineered anti-CD20 mAb) was studied in a phase I trial following a 3 + 3 dose escalation design [143]. Ublituximab was administered weekly for the first two cycles and then on day 1 of cycle numbers 4, 6, 9, and 12 while TGR-1202 was administered daily. There were 12 patients with CLL included in the trial. Reported toxicities were 44% day 1 infusion reactions, 41% neutropenia, 34% diarrhea, and 28% nausea; no grade 3 or 4 toxicities were seen. There were no cases of hepatotoxicity. In the ten patients with CLL, there was a median progression-free survival of 8 months. Enrollment continues in the higher dose cohort.

The combination of ublituximab with TGR-1202 and ibrutinib has also been administered to patients with B-cell malignancies [144]. Ublituximab was dosed at 900 mg weekly for the first two cycles and then on day 1 of cycles 4, 6, 9, and 12. TGR-1202 was dose escalated at 400, 600, 800, and 1200 mg while the ibrutinib dose was held stable at 420 mg for patients with CLL and 560 mg for patients with NHL. There were three patients with CLL and SLL included. Of the adverse events, 20% of patients experienced day 1 infusion reactions with no grade 3 or 4 reactions noted, while 20% of patients experienced neutropenia which was grade 3 or 4, and 30% experienced diarrhea, constipation, or fatigue with no grade 3 or 4 events. The overall response rate was 86% with two of three patients with CLL/SLL responding. Based on these findings, phase II studies are planned.

Adoptive Cellular Therapy

New cellular therapies including CAR-T cells are being developed for patients with B-cell malignancies including patients with CLL [145]. CAR-T cells can be associated with adverse events including neurotoxicity and cytokine release syndrome. Experience with managing the toxicities of these therapies is critical for safe delivery of these therapies.

Targeting Minimal Residual Disease in CLL

In patients achieving complete remission after therapy for CLL, those who achieve MRD-negative CR tend to have responses that last longer and also have better OS. Four-color flow cytometry or allele-specific oligonucleotide PCR (ASO-PCR) with a sensitivity of detecting 1 CLL cell in 10,000 leukocytes is recommended for use in clinical trials reporting on MRD eradication.

Disease-Related Complications of CLL

Immune-Mediated Cytopenias in CLL

Autoimmune hemolytic anemia (AIHA), autoimmune thrombocytopenia (AIT), and pure red cell aplasia (PRCA) develop in some patients with CLL. The incidence of AIHA is 4–11% [6, 146, 147] and that of AIT 2–3%. PRCA is least common. Fludarabine has been associated with AIT and AIHA [148]. Prednisone is the usual treatment for AIHA and AIT, with a high likelihood of response initially. However, more than 60% of patients relapse when treatment is stopped. Intravenous immunoglobulin produces response in 40% of patients, but these responses tend to be transient. Cyclosporine A is another option for treatment of immune-mediated cytopenias and can produce responses even in patients with steroid refractory immune cytopenias [149]. Rituximab, alemtuzumab, and the combination of rituximab, cyclophosphamide, and dexamethasone [150–152] have also been used to treat autoimmune complications of CLL.

Hypogammaglobulinemia

Hypogammaglobulinemia is a frequent complication of CLL. Because of the high cost of therapy and its limited activity in preventing serious infections, monthly intravenous gammaglobulin replacement therapy is usually limited to hypogammaglobulinemic patients who experience repeated sino-pulmonary bacterial infections.

Transformations

Richter's Syndrome

The term Richter's syndrome (RS) refers to the development of large-cell lymphoma (LCL) during the course of CLL. RS is usually associated with worsening systemic symptoms including B symptoms, elevated LDH, rapid tumor growth, and/or extranodal involvement. Diagnosis requires tissue biopsy. PET scanning helps in identifying sites to direct tissue biopsy. Gene rearrangement studies and isotype analysis suggest that the CLL and LCL cells frequently share identical clonal origins. The LCL is usually resistant to therapy, and the median survival of patients who develop RS is approximately 6–9 months [153, 154]. If the gene rearrangement studies do not show the identical clonal origin, then the LCL should be treated as independent of the history of CLL and the prognosis can be excellent [155]. The presence of NOTCH mutations increases the risk of transformation. The presence of NOTCH mutation was associated with a risk of development of DLBCL in 30% of patients at 10 years [156].

Prolymphocytic Transformation

The NCIWG criteria allow a diagnosis of CLL to be made in the presence of ≤55% prolymphocytes. The presence of prolymphocytes >55% indicates prolymphocytic transformation.

Prolymphocytic Leukemia

Prolymphocytic leukemia (PLL) is characterized by splenomegaly, a high number of circulating prolymphocytes, minimal lymphadenopathy, and a median survival of less than 3 years. Prolymphocytes are larger and less homogenous than CLL cells, and have abundant clear cytoplasm, clumped chromatin, and a prominent nucleolus. Prolymphocytes can be of either B- or T-cell type. B-PLL cells usually do not express CD5 but stain strongly for surface immunoglobulin and FMC-7. TP53 mutations and 11q23 or 13q14 deletions are common in B-PLL [157–159]. Approximately 20% of cases of PLL are of T-cell phenotype. Over 70% of T-PLL shows overexpression of the oncoprotein TCL-1 [160, 161].

Splenectomy and lymphoma-like regimens have been used to treat PLL without much success. In a study at MDACC, a 38% ORR (18% CR) was seen with a 5-day schedule of fludarabine administered every 4 weeks. Dearden et al. reported an ORR of 48% with pentostatin (2′ deoxycoformycin) [162]. Alemtuzumab (Campath-1H) also has shown promising activity in T- and B-PLL [163–165] with an ORR of 51%, CR rates of up to 39.5%, and median survival of 7.5 months. In a study from Royal Marsden Hospital, alemtuzumab (Campath-1H) was administered intravenously three times weekly to patients with previously treated T-PLL until maximal response [166]. The ORR was 76% with 60% CR. However, responses with alemtuzumab are short-lasting and disease progression is the norm. Ibrutinib and idelalisib may have a role in this disease.

Conclusion

Based on the advances made in our understanding of the biology of CLL, there are now many avenues for investigation for novel therapies. Given that these newer therapies are generally better tolerated than chemoimmunotherapy, many more patients are eligible for therapy than in the past. This will hopefully lead to a prolongation in remissions in these patients while maintaining a good quality of life.

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Hairy Cell Leukemia

10

Sonia Ali and Alan Saven

Introduction

The WHO categorizes hairy cell leukemia (HCL) as a mature B-cell neoplasm. HCL is characterized by lymphocytes with prominent cytoplasmic projections (hairy cells) infiltrating the bone marrow and spleen, leading to pancytopenia, bone marrow fibrosis, and splenic enlargement. Hairy cells have a unique immunophenotypic profile—CD11c+, CD20+, CD25+, and CD103+—that confirms its diagnosis. The course of HCL is usually chronic, but can often be progressive, and most patients require treatment at some point. The purine nucleoside analogues, pentostatin and cladribine, are highly active, but cladribine is the preferred first-line choice due to its efficacy, brief treatment duration, and favorable toxicity profile. Other therapeutic options include rituximab, interferon-alpha, vemurafenib, and splenectomy. With current therapy, an overall survival of 87% at 12 years has been reported.

History

HCL was originally recognized in the 1920s but was not identified as a unique entity with distinct pathological and clinical characteristics until 1958 when Bouroncle and colleagues characterized it as *leukemic reticuloendotheliosis* [1] and described the first 26 cases. In their landmark article, the authors provided a comprehensive description of the clinical course, pathology, and limited treatment at the time with alkylating agents and splenectomy. The term "hairy cell leukemia" was first coined by Schreck and Donnelly in 1966 when they noted hairlike cytoplasmic projections on phase-contrast microscopy [2]. The last 50 years, and especially the last two decades, have been spent defining HCL as a B-cell neoplasm [3, 4] and have heralded dramatic therapeutic advances with the purine nucleoside analogues.

Epidemiology and Etiology

HCL is uncommon and accounts for 2–3% of all adult leukemias in the USA [5]. According to the Surveillance Epidemiology and End Results (SEER) database, 2856 cases were diagnosed between 1978 and 2004 [6]. There is a 4:1 male predominance and the median age at presentation is 50 years [5]. New data suggest a bimodal incidence pattern, with an early peak around age 40 years and a later peak at 80 years [6]. The disease is more common in Caucasians, with an increased incidence in Ashkenazi Jewish men.

No well-defined etiology for HCL has been reported. Case reports have suggested an association with farming, woodworking, and exposure to organic solvents [7]. A recent hospital-based case-control study in France noted significant associations between HCL and organochlorine insecticides, and phenoxyacetic and triazine herbicides, though the numbers in the study were small [8]. Infectious etiologies such as EBV and HTLV-1 have also been postulated as causes [9, 10]. Familial cases of HCL have been rarely reported. Makower et al. described two cases of familial HCL. In one case, a 50-year-old man developed HCL and a year later his mother was diagnosed with the same entity. In the other family, an aunt of a patient with HCL was diagnosed with Hodgkin's disease. Interestingly, in both families, the younger generation developed the hematologic malignancy at an earlier age. This phenomenon, known as anticipation, has been noted in other malignancies [11]. Cases of familial HCL have also identified HLA haplotypes specific to each family. Each family's HLA haplotype was unique and there has been no identification of a common HLA haplotype among unrelated cases of HCL [11, 12].

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Pathogenesis

Ontogeny

With the advances in molecular techniques, the ontogeny of HCL is becoming clearer. The hairy cell phenotype is that of a late B-cell precursor, likely an activated memory B cell, with aberrant gene expression [13]. The post-germinal center origin is supported by the presence of Bcl-6 mutations and somatic point mutations in the immunoglobulin variable region of the heavy chain [14, 15]. Furthermore, hairy cells express several pan B-cell markers including CD19, CD20, and CD37, but are devoid of the early markers of B-cell development, including CD21 and CD 24 [16]. Hairy cells express the plasma cell antigen-1 (PCA-1) but lack expression of PC-1 which appears later in B-cell ontogeny. This observation suggests that hairy cells do not differentiate into terminal B cells, i.e., plasma cells [3]. DNA microarray analysis illustrates a homogeneous phenotype distinct from other B-cell malignancies. When compared to normal B cells, hairy cells share many genes with memory B cells involved in proliferation and apoptosis [4].

Adhesion/Homing

Hairy cells are highly adherent and can spontaneously bind to several matrices, including fibronectin, vitronectin, and hyaluronan [17, 18]. This binding is facilitated by specific adhesive proteins on hairy cells, including the integrins α4β 1, α5β1, and αvβ3 [18]. Hairy cells characteristically disseminate into the red pulp of the spleen and hepatic sinusoids and portal tracts, but spare lymph nodes [19]. Not only do hairy cells infiltrate many different types of tissues, but they also modify the tissues they infiltrate. Thus, they cause bone marrow fibrosis and form vascular lakes (pseudosinuses) in the spleen [20]. This modification is inherent to the tissue matrix and is enhanced by hairy cell interactions [18]. For example, fibronectin is important in the development of bone marrow fibrosis and it is thought that hairy cells themselves are intricately involved in its production and assembly [17]. Recently, gene analysis has provided more insights into hairy cell adhesion and targeting. For instance, the lack of hairy cell lymph node infiltration can be explained by downregulation of CCR7, a chemokine receptor that allows B cells to enter lymph nodes. Also, hairy cells remain confined to blood-related compartments due to upregulation of genes that prevent their extravasation [4].

Cytogenetics

No karyotypic abnormality is pathognomonic for HCL. Clonal karyotypic abnormalities are variable and range from 20 to 67% of patients [21]. Unlike most other B-cell malignancies, HCL lack balanced chromosomal translocations which occur with immunoglobulin gene rearrangements that are switched off in memory B cells [13]. Instead, chromosomal gains, deletions, and inversions have been identified. In one study, 40% of karyotypic abnormalities involved chromosome 5, with aberrations in band 5q13 being most common [21]. Other chromosomal abnormalities include deletion of 14q and losses of the long arm of chromosome 7 [22, 23]. Evaluation by FISH has revealed that p53 deletions, a marker found in aggressive disease, occur in HCL. The clinical significance of this finding in an indolent disease is currently under investigation [24].

Diagnosis

Histopathologic and morphologic evaluation of the bone marrow is key to establishing the diagnosis of HCL [25]. Classical cytochemical stainings such as tartrate-resistant acid phosphatase (TRAP) have generally been supplanted by modern diagnostic techniques of flow cytometry and immunohistochemical (IHC) staining.

Cytology

Hairy cells are uniform and monotonous in their appearance [25]. A typical hairy cell is slightly larger than a mature lymphocyte with a distinct nucleus that is usually ovoid, but can also be slightly indented [25]. Unlike other B-cell malignancies, the chromatin is uniformly granular without clumping [26]. Morphologically, hairy cells display features suggestive of a metabolically active cell [27]. They have variable amounts of blue-gray cytoplasm and abundant mitochondria and ribosomes. Hairy cells exhibit thin cytoplasmic "hair-like" projections often appearing as serrated borders (Fig. 10.1). Phase-contrast microscopic studies of live cells show that the surface of these cells is in a constant state of change, reflecting ongoing cytoskeletal and signaling activity [13, 28].

Rarely, ribosomal lamellar complexes, or broad-shaped inclusions, can be seen in the cytoplasm on electron microscopy. These organelles are thought to originate from the endoplasmic reticulum and are characterized by alternating layers of ribosome-like granules and fibrous lamellae [29, 30]. Present in half of the cases, the ultrastructural inclusions are not unique to HCL and have been noted in other lymphoid malignancies [30]. They are of unclear clinical significance [31].

Hairy cell cytoplasm stains strongly for TRAP [32]. Isoenzyme 5 acid phosphatase present in hairy cell cytoplasm resists decoloration with tartrate [33]. Most other lymphoid cells, monocytes, and myeloid cells stain variably for

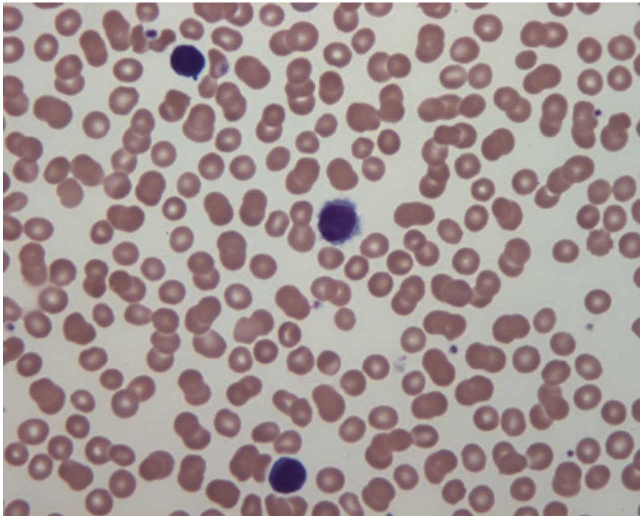

Fig. 10.1 Peripheral blood smear from a patient with HCL. The hairy cell is slightly larger than a mature lymphocyte with ovoid nuclei. Hairy cells characteristically have abundant, *gray-blue* cytoplasm with thin "hairlike" projections (×1000) (corresponds to figure pb 1000×)

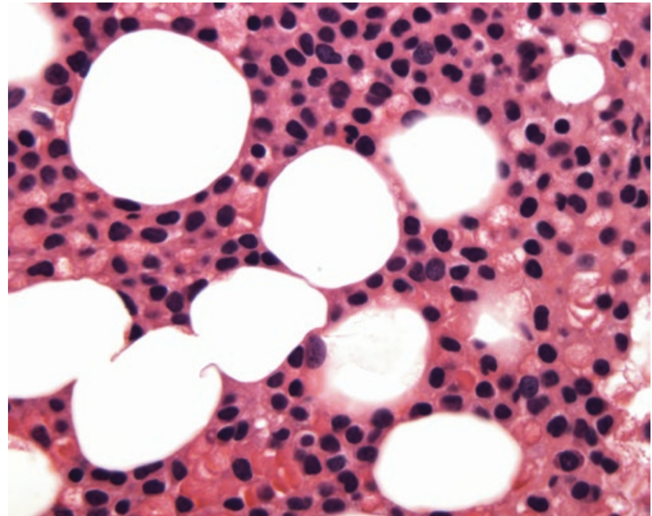

Fig. 10.2 Hairy cell leukemia in the bone marrow, characterized by well-spaced lymphocytes with a "fried-egg" appearance due to the distinct round-to-oval nuclei, which are centrally placed within a pale-staining cytoplasmic domain (×1000) (corresponds to figure bm 1000×)

acid phosphatase activity in the absence of tartrate [16]. TRAP staining is labor intensive and difficult to perform in paraffin-embedded tissues and it is rarely used in the era of immunophenotyping.

Histopathology

Blood and Bone Marrow

Abnormalities in the hemogram are classically seen at presentation in HCL patients [26]. Pancytopenia is common and reported in 80% of patients. Leukopenia is frequently noted [5]. Circulating monocytes are usually absent from the peripheral blood. Despite findings of marrow fibrosis, leukoerythroblastosis is not seen. Circulating hairy cells are variable and oftentimes very difficult to identify [26].

Bone marrow involvement is seen in nearly all patients with HCL [34]. It is often difficult or impossible to obtain an aspirate [25]. The biopsy can show a hypercellular picture. Hairy cells demonstrate patchy or diffuse infiltration of the marrow. A closer examination of the infiltrate reveals a distinctive wide-spaced separation of cells with a surrounding halo, often referred to as a "fried-egg" appearance (Fig. 10.2) [34]. This loose packing of cells results from hairy cells adhering to the reticulin–fibronectin network. Few fibroblasts are seen and trichrome staining does not show deposition of mature collagen [26]. The residual hematopoietic tissues exhibit nonspecific changes [34]. Other collection of cells including small lymphocytes, plasma cells, and mast cells is often identified. Not uncommonly, HCL produces a hypocellular marrow which can be difficult to distinguish from aplastic anemia. Immunostains with CD20 may be helpful (see Fig. 10.3) [35].

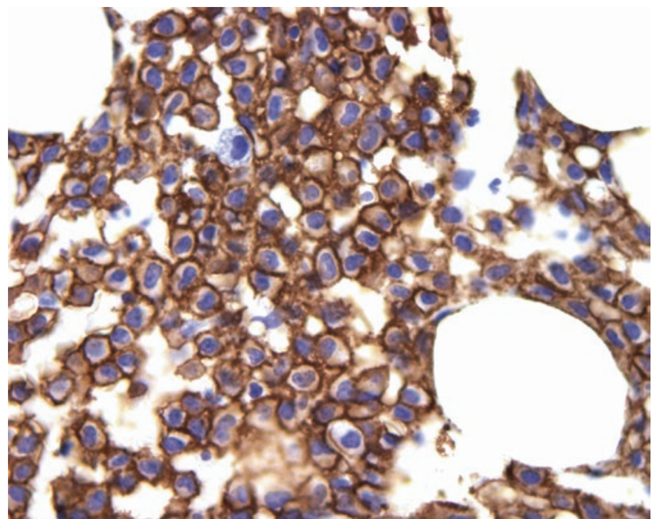

Fig. 10.3 Immunoperoxidase staining with anti-CD20 (B-cell marker), demonstrating strong membrane positivity (×1000). This stain is very useful in evaluating MRD in bone marrow specimens (corresponds to figure bm cd20 1000×)

Spleen and Liver

Splenic sequestration of hematopoietic elements is a characteristic feature of HCL [36]. HCL mostly affects the red pulp. On microscopy, there is a heavy infiltration of monotonous

cells in the expanded red pulp, sometimes making the individual cords and sinuses indistinguishable. The white pulp atrophies overtime [36]. Hairy cells replace endothelial cells that line the splenic sinusoids and merge to form congested splenic lakes, often appearing as hemangiomas [20]. Remodeling is thought to occur when hairy cells directly network with endothelial cells via integrin receptors and the vitronectin matrix of the basement membrane [13]. Such splenic findings are striking and can sometimes be seen in the bone marrow.

Similarly in the liver, hairy cells infiltrate the hepatic sinuses and portal tracts but spare the parenchyma. They also form characteristic lesions but appear more as angiomas than pseudosinuses since they lack circumferential ring fibers [20].

Genetic Features

With the use of whole-exome sequencing, Tacci et al. recently detected the BRAF V600E mutation in an entire cohort of 48 HCL patients. The absence of this variant in 195 patients with other peripheral B-cell lymphomas or leukemias established it as a key genetic lesion in HCL [37]. The oncogenicity of this mutation results from constitutive activation of the RAF-MEK-ERK mitogen-activated protein kinase pathway. Subsequent studies have confirmed the presence of the BRAF V600E mutation in HCL, with two exceptions [38, 39]; the molecular variants HCL-variant [38] and HCL with IGHV4–34 immunoglobulin rearrangement [39] lack this mutation. Thus, while distinct in the indolent lymphoproliferative disorders, the BRAF V600E mutation has yet to be incorporated into the diagnostic criteria for HCL.

Downstream of BRAF is MEK1, a dual-specificity kinase encoded by MAP 2 K1 (mitogen-activated protein kinase kinase 1). Mutations of the MAP 2 K1 gene have recently been identified in classical, variant, and IGHV-34-expressing HCL patients and appear to be mutually exclusive of the BRAF V600E mutation [40]. Aside from isolated reports [41, 42], MAP 2 K1 mutations in other hematologic malignancies have not been reported. This seemingly unique mutation makes it an attractive target for therapeutic manipulation and warrants further investigation.

Immunophenotyping: Flow Cytometry

Hairy cells can be identified by multicolor flow cytometry to a high degree of certainty even when they compose less than 1% of circulating lymphocytes [43, 44]. They display a mature B-cell phenotype and express pan B-cell markers including CD19, CD20, CD22, and CD 79A [16]. One or more heavy chains and a single light chain are displayed on the cell surface [45, 46]. Frequently, hairy cells demonstrate the presence of surface IgG, specifically the IgG3 isotype, and do not undergo normal B-cell differentiation with class switching [47]. Three markers of importance in the characterization of HCL include CD11c (common in myelomonocytic cells), CD25 (the IL-2 receptor), and CD103 (the alpha subunit of the alpha-beta integrin in intraepithelial T cells) [48–50]. Though these markers are not limited to HCL and can be seen in other lymphoproliferative disorders, such as splenic marginal zone lymphoma (SMZL), their co-expression is unique. For instance, CD11c is distinguished from other disorders by its nearly 30-fold higher intensity of expression in HCL [47, 49]. Moreover, CD103 has the greatest sensitivity and specificity for HCL [26, 51]. Researchers have evaluated the predictive value of the composite phenotype of these antigens. A scoring system was developed by the Royal Marsden Group using the markers: CD11c, CD25, CD103, and HC2 (HCL-associated antigen involved in cell differentiation). Ninety-eight percent of the evaluated cases of HCL had a score of 3 or 4 [52]. Also of note, primarily due to its potential therapeutic implications, is CD52, a marker that has recently been identified in both variant and classical HCL [53].

Further distinctions between variant and classical HCL can be made via flow cytometry. CD123, which is the alpha chain of the IL-3 receptor, is positive in the majority of classical HCL and dim or negative in variant HCL [54–56]. Additionally, CD25 has been shown to be commonly absent in HCL variant [56].

Immunophenotyping: Immunostains

Monoclonal antibodies with specificity for HCL are useful diagnostic tools. They can be performed easily in peripheral blood and paraffin-embedded tissues, and are thus valuable in the evaluation of minimal residual disease (MRD) in treated patients [16]. In addition to the routine B-cell markers like CD20 and PAX5, specific markers for HCL include TRAP, DBA.44, and cyclin D1 [27, 57, 58]. DBA.44 recognizes an unknown fixation-resistant B-cell antigen that is expressed in mantle zone lymphocytes, reactive immunoblasts, and monocytoid B cells [57]. It reacts strongly with HCL (Fig. 10.4) [59]. Although DBA.44 is expressed in other low-grade B-cell lymphoproliferative disorders, a recent study suggests that the combination of DBA.44/TRAP staining has a 97% specificity for HCL [60]. Moreover, CD20 immunostaining is a useful marker in quantifying disease, as it often highlights HCL infiltrates not detected on routine hematoxylin and eosin staining [61].

Annexin A1 (ANXA1) has been identified as a gene that is upregulated in HCL. One study evaluated samples of 500 B-cell tumors for the anti-ANXA-1 monoclonal antibody and found the assay to be both highly sensitive and specific

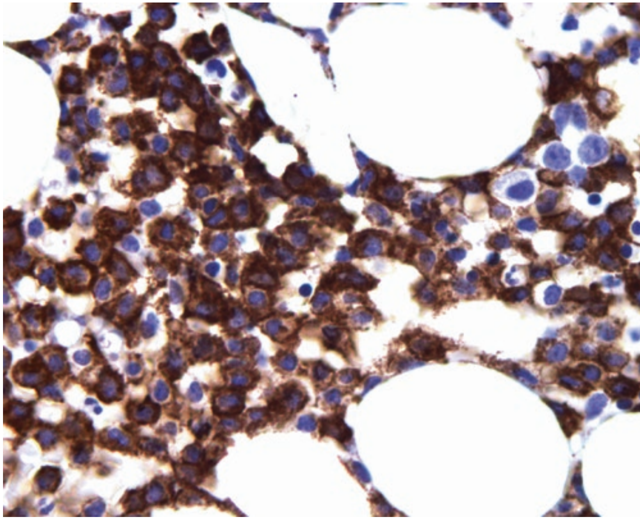

Fig. 10.4 Immunoperoxidase staining with DBA.44. in bone marrow of HCL patient. DBA.44 reacts strongly with HCL. A combination of DBA.44/TRAP staining has a 97% specificity for HCL (corresponds to figure dba-44 1000×)

for HCL (100%) [62]. This precision was not reproduced in a subsequent study, in which only 74% of HCL cases stained positive for ANXA1. Interestingly, none of the HCL variant or BRAF V600E-mutated HCL cases stained for ANXA1 [56].

Clinical Features

General

The onset of HCL may be insidious and its course chronic. It is characterized by pancytopenia and in particular monocytopenia, splenomegaly, and impaired immunity without significant lymphadenopathy [36]. This unique clinical presentation reflects the leukemic infiltration of hairy cells in the bone marrow, spleen, and liver.

In the original description of HCL, fatigue and weakness were the most common symptoms on initial presentation [1]. Also, frequently noted are symptoms of an opportunistic infection and abdominal fullness from splenomegaly. Some patients are incidentally found on physical examination or laboratory workup [5].

On physical examination, splenomegaly is the most prominent finding seen in 80–90% of patients. Spleen size may be variable, but sometimes can be massive [34]. Older studies have suggested that massive splenomegaly, along with patient age and hemoglobin concentration, is associated with a worse prognosis [63]. When present, hepatomegaly usually accompanies splenomegaly, and is seen in 50% of patients [5]. Palpable peripheral lymphadenopathy, unlike other chronic lymphoproliferative disorders, is not common [64]. Internal adenopathy is recognized in one-third of patients with HCL and is thought to be related to disease duration and may correlate with overall survival [64, 65].

Infectious Complications

Infections are a common complication in HCL and a cause of death throughout its course [36, 45]. Among multiple case series, the incidence of serious infections has ranged from 20 to 47%, which includes pneumonia and septicemia [36, 66, 67]. Pyogenic organisms consist of *Pseudomonas aeruginosa*, *Escherichia coli*, and *Enterococcus* species [68]. A higher frequency of intracellular organisms such as *Legionella pneumophila* and *Mycobacterium kansasii* has also been noted and thought to arise from defects in monocytes and decreased dendritic cells [45, 69]. Multiple studies have chronicled neutropenia and monocytopenia as contributing causes of the immunodeficiency in HCL [5]. A study of 73 long-term patients found that baseline lymphopenia may be a prognostic factor of increased risk of infectious complications [67].

Secondary Malignancies

Patients with HCL are at increased risk of secondary malignancies [70–72]. Secondary cancers have been attributed to decreased T-cell function from treatment as well as immunologic aberrations from the underlying disease itself [70, 73, 74]. In their 20-year experience with HCL, Wing et al. noted that 22% of their 117 patients developed second malignancies. Cancer risk peaked at 2 years after the diagnosis of HCL and then steadily declined [73]. The authors in this study conclude that HCL patients may be prone to secondary malignancies from the HCL tumor burden rather than genetic predisposition or the immunosuppressive effects of treatment.

Long-term data suggest that secondary cancers are only moderately increased with exposure to purine nucleoside analogues. In their extended follow-up of HCL patients treated with cladribine at Scripps Clinic, Goodman et al. noted 58 second malignancies in 379 treated patients [75]. A subsequent study at the same institution evaluated 83 patients ≤40 years with HCL treated with cladribine; though the excess frequency of developing a second primary malignancy was 1.60 (95% confidence interval, 0.80–2.89), it was not statistically significant [76]. In a retrospective analysis of 487 patients with HCL treated with purine nucleoside analogs, Cornet et al. reported an increased incidence of second malignancies, especially hematological malignancies (standardized incidence ratio 1.86, CI 1.34–2.51 for all malignancies; 5.32, CI 2.90–8.92 for hematological malignancies) [74]. The National Cancer

Institute (NCI) quantified second cancer incidence and cause-specific mortality among 3104 survivors of HCL between 1973 and 2002. They found that the rate of second cancers was 32% compared to the expected 23% in the general population [70].

Other

Extremely rare manifestations of HCL include cutaneous, bone, serosal, and meningeal involvement [77]. Hypocholesterolemia and elevated liver function tests are disease-related findings in HCL [78, 79]. Polyclonal and monoclonal gammopathies have also been noted in 3–20% of patients and can be associated with plasma cell disorders, lymphoma, or autoimmune processes. Autoimmune-associated disorders include polyarthritis nodosa and leukocytoclastic vasculitis [78, 80].

Differential Diagnosis

HCL must be distinguished from other chronic lymphoproliferative disorders that present with splenomegaly and cytopenias, such as hairy cell leukemia variant (HCL variant), splenic lymphoma with villous lymphocytes, and prolymphocytic leukemia. This distinction is critical since these different disorders have unique management approaches and respond quite differently to treatment with interferon-alpha and purine nucleoside analogues.

The differential diagnosis is based on morphologic and phenotypic criteria (Table 10.1).

Hairy Cell Leukemia Variant

HCL variant is a very rare B-cell lymphoproliferative disorder with features distinct from HCL. Patients with HCL variant

Table 10.1 HCL differential diagnosis

Lymphoid malignancy	Clinical characteristics (age/sex)	Morphology	Peripheral blood count	Immunophenotype	Genetics	Spleen	Survival
HCL	Median age: 50 years	Cytoplasm: Irregular	Neutropenia and lymphopenia	CD11c:+++	BRAF V600E mutation ++/–	Red pulp	87% at 12 years
	Male predominance: 4:1	Nucleus: Reniform	Monocytopenia:+	CD25: ++			
		Nucleolus: Not present		CD 103:+++			
				TRAP: ++			
				CD 123 ++			
				ANXA1 +/–			
HCL-variant	Median age: 80 years	Cytoplasm: Irregular	Lymphocytosis	CD11c:++	BRAF V600E mutation ––/––	Red pulp	Median: 9 years
	Male predominance: <2:1	Nucleus: Round	Monocytopenia:–	CD25: ––/–			
		Nucleolus: Present		CD 103:++			
				TRAP: +/–			
				CD 123 +/––			
				ANXA1 –/–			
SMZL	Median age: 65 year	Cytoplasm: Irregular	Lymphocytosis	CD11c:+		White pulp	80% at 5 years
	No gender predominance	Nucleus: Round	Monocytopenia:–	CD25: +/–			
		Nucleolus: Often present		CD 103:+/–			
				TRAP: +/–			
				CD123 +/–			
				ANXA1 –/–			
CLL	Median age: 70 years	Cytoplasm: Smooth	Lymphocytosis	CD11c:+/–			Median: 3 years
	Male predominance: <2:1	Nucleus: Round	Monocytopenia:–	CD25: –			
		Nucleolus: Present		CD 103: –			
				TRAP: –			

+ present, – absent
HCL hairy cell leukemia, *SMZL* splenic marginal zone lymphoma, *CLL* chronic lymphocytic leukemia

are often diagnosed in their seventh or eighth decades and unlike HCL lack a strong male predominance. They typically present with splenomegaly and high leukocyte counts. Though cytopenias may be noted, neither monocytopenia nor neutropenia is a feature of HCL variant [81, 82]. The bone marrow and splenic histologies are similar to those of HCL [81]. Morphologically, cells of HCL variant are intermediate between HCL and B-cell prolymphocytic leukemia [83]. The cells lack monotony and are more varied in appearance. The nucleus is well circumscribed with a prominent nucleolus similar to that of prolymphocytes, while the cytoplasm is more basophilic [81]. Akin to the morphology, the diagnostic profile for HCL variant can be quite distinct. TRAP staining is variable and often negative. Considering the three markers that are characteristically expressed in HCL, CD11c is strongly positive, CD103 is positive in 60% of cases, and CD25 is negative [83, 84].

Examination of the immunoglobulin heavy-chain (IGH) rearrangements and somatic hypermutation patterns has noted significant differences between HCL and HCL variant. In fact, the mutation status of HCL variant mirrored splenic marginal zone lymphoma (SMZL) more than HCL. Specifically, IGHV4–34 was overrepresented in patients with HCL variant and SMZL [85]. Exploration of IGHV4–34 overexpression has established it as a distinct and separate entity from HVL variant, predicting an even worse prognosis [86, 87].

With an aggressive clinical course and refractoriness to traditional therapy, the median survival of patients with HCL variant is 9 years [84]. In addition, approximately 5–10% of patients have transformation to a large-cell process, characterized by significant leukocytosis, B symptoms, and an overall poor prognosis [88]. Moreover, a Japanese variant of HCL has also been described with large granular lymphocytosis [89].

Splenic Marginal Zone Lymphoma

SMZL is a chronic B-cell lymphoproliferative disorder characterized by splenomegaly and lymphocytosis with more polar villous projections. These cells are smaller than hairy cells and have more condensed chromatin [90]. When the hairy cell scoring system is applied to SMZL, the score is usually low [52]. CD103 is rarely positive in SMZL, and in most studies, CD25 and CD11c were positive in 25–47% of cases [91]. The immunologic profile of SMZL is very similar to that of HCL variant and can present a diagnostic challenge. Histologically, SMZL can be distinguished from HCL and HCL variant by splenic expansion of the white pulp and appearance of nodularity in the bone marrow [26].

SMZL usually has a more indolent clinical course with reported 5-year overall survivals of 80% [90].

B-Cell Prolymphocytic Leukemia

B-cell prolymphocytic leukemia is frequently noted in elderly males with prominent splenomegaly without significant lymphadenopathy [92]. The presenting WBC is elevated and often greater than 100×10^9/L with predominant prolymphocytes [93]. Like SMZL, B-cell prolymphocytic leukemia shares many similarities with HCL variant. B-prolymphocytes are larger lymphocytes with a condensed chromatin and a prominent central nucleolus. Immunophenotypically, they are CD25, CD103, and CD11c negative. Overall, they have a poor prognosis and their median survival is 3 years [94].

Treatment

General

Though HCL is an indolent disease, most patients ultimately require treatment [66]. Generally, patients are treated for worsening cytopenias (hemoglobin <10 g/dL, platelet count $<100 \times 10^9$/L, or absolute neutrophil count $<1.0 \times 10^9$/L), infectious complications, and symptomatic splenomegaly. Other less common reasons include bulky lymphadenopathy, progressive visceral or bony disease, or significant autoimmune processes.

Purine Nucleoside Analogues

For many years, splenectomy and interferon-alpha were the standard therapeutic approaches to HCL. The purine nucleoside analogues, cladribine [2-chlorodeoxyadenosine (2-CdA)] and pentostatin (2′-deoxycoformycin), came into clinical use in the mid-1980s and are considered to be the cornerstone of HCL therapy. The discovery that adenosine deaminase (ADA) deficiency produced lymphopenia in children with combined immunodeficiency syndrome led to the development of purine nucleoside analogues [95]. ADA is the major pathway for deoxypurine nucleoside degradation. Resistance to or inhibition of ADA can lead to a buildup of intracellular purine nucleotides which are very toxic to lymphocytes [96–98]. Cladribine (substrate analogue) and pentostatin (direct inhibitor) were developed to oppose the action of ADA.

Cladribine (2-CdA)

Cladribine is commonly chosen as the initial therapy because of its brief treatment administration and high, durable response rates. As a purine nucleoside analogue, it is resistant

to ADA. Cladribine accumulates in lymphoid cells because they have high levels of deoxycytidine kinase [98]. This enzyme phosphorylates cladribine, creating a deoxynucleotide which is then incorporated into DNA, thereby inducing DNA strand breaks and inhibiting repair. Cladribine's potency in indolent lymphomas is a function of its cytotoxicity to both dividing and nondividing lymphocytes [99].

Cladribine was first reported to be effective for HCL in 1990. Under the leadership of Ernest Beutler, investigators at Scripps Clinic first reported on 12 HCL patients treated with a single 7-day course of cladribine at 0.1 mg/kg/day by continuous intravenous infusion. Of those 12 patients, 11 achieved a complete response and the responses were maintained for 16 months [100].

The largest single-institution series was at Scripps Clinic and evaluated 349 previously treated and untreated HCL patients [71]. After a median follow-up of 52 months, 91% of patients achieved a complete response and 7% a partial response. Ninety patients (26%) had relapsed at a median of 29 months. The median survival rate at 48 months was 96%. Rosenberg et al. subsequently reported on 83 patients aged 40 years or less [76]. After a median follow-up of 251 months, 88% of patients achieved a complete response and 12% a partial response. Forty-five (54%) of patients who achieved a response ultimately relapsed at a median of 54 months. Median overall survival for all patients following the first cladribine course was 231 months. The authors hypothesized that the variation in survival data may be attributable to the intrinsic biologic differences between young and old HCL patients.

In the 25-year interval since the introduction of cladribine, many studies have acquired long-term patient data (Table 10.2). Among the assessable patients with HCL treated with cladribine as a single 7-day continuous infusion, complete responses have ranged between 76 and 100%. The majority of these patients have enjoyed long-term remissions with relapse rates of 14% at 24 months and 36% at 9.7 years [101, 102]. In one of the longest follow-up studies, 85% of the patients were alive at 20.9 years [76].

Standard cladribine dosing is a 7-day continuous infusion at a dose of 0.1 mg/kg/day. Alternative treatment schemes have been developed in the hopes of ameliorating prolonged myelosuppression and obviating the need for a pump. Alternative schedules have included a 5-day 2-h infusion at a dose of 0.14 mg/kg/day, weekly 2-h intravenous infusion, subcutaneous administration, and oral administration. Several studies have shown that the 2-h 5-day infusion is equally efficacious with a similar toxicity profile [111]. Robak et al. conducted a prospective study of 132 patients, comparing cladribine administered in a weekly versus daily schedule. Patients were randomized to receive either cladribine 0.12 mg/kg as a 2-h intravenous infusion daily for 5 days or 0.12 mg/kg in a 2-h intravenous infusion once a week for 6 weeks. Results of the trial showed similar complete remission rates, progression-free survival, and overall survival between the two groups. Despite prior reports showing improvement in infectious complications with the weekly dosing, there was no significant difference in grade 3 or 4 infections [112]. Similar results were noted in a Swiss study that compared subcutaneous daily 2-CdA with weekly treatment [113]. Though treatments with these alternative schedules appear promising, they lack the support of long-term follow-up, and the 7-day continuous infusion and 2-h 5-day infusion of cladribine are both considered standard.

Neutropenic fever is the principal acute toxicity of cladribine therapy in HCL, occurring in 42% of treated patients [71]. Infectious complications include bacterial and opportunistic infections. Immunosuppressive effects of

Table 10.2 Long-term follow-up studies with cladribine

Study	Patients (no.)	Median F/U (years)	Initial complete remission rate (%)	Relapse rate (%)	Median time to relapse (months)	Overall survival
Seymour et al. [103]	46	2.5	78	20	16	NA
Hoffman et al. [104]	49	4.6	76	20	NA	95% at 4.6 years
Goodman et al. [75]	209	7	95	37	42	97% at 9 years
Jehn et al. [105]	44	8.5	98	39	48	79% at 12 years
Chadha et al. [106]	86	9.7	79	36	35	87% at 12 years
Else et al. [101]	45	16	76	38	NA	100% at 15 years
Rosenberg et al. [76]	83	20.9	88	54	54	85% at 20.9 years
Lopez et al. [107]	80	5.2	88*	25	NA	NA
Cornet et al. [74]	281	4.4	83	18	NA	NA
Hacioglu et al. [108]	78	2.3	81	17	24	96% at 2.1 years
Somasundaram et al. [109]	27	2.2	100	18	48	96% at 2.2 years
Ruiz-Delgado et al. [110]	11	2.1	100	27	NA	91% at 11 years

NA data not available
aafter a second course of cladribine in some patients

cladribine can persist for extended periods with decreases in CD4+ lymphocytes [103]. Herpes zoster is the most frequently reported late infection [75]. Granulocyte colony-stimulating factor (G-CSF) was evaluated in patients treated with cladribine therapy. Although G-CSF ameliorated neutropenia, it did not improve rates of neutropenic fever or hospital admissions for antibiotics and is thus not routinely recommended [114].

Pentostatin (2′-Deoxycoformycin)

Pentostatin is a natural product that is derived from *Streptomyces antibioticus*. Unlike cladribine, it irreversibly inhibits ADA and leads to the accumulation of cytotoxic metabolites. Pentostatin was first described to be an effective agent against HCL in the mid-1980s [115].

One of the largest studies evaluating its efficacy randomized 313 patients to pentostatin or interferon-alpha-2a for 6–12 months. This study used a crossover design where patients in the interferon arm could cross over to pentostatin upon progression. In the initial results, 76% of pentostatin patients achieved a complete remission compared to only 11% treated with interferon-alpha. In patients who crossed over from initial interferon to pentostatin, the complete response rate was 66% [63]. This study underscores the benefits of pentostatin both in treated and untreated patients. Flinn et al. reported on the long-term data from this trial with a median follow-up duration of 9.3 years. The relapse rate was 18% and included both patients initially treated with pentostatin and those who crossed over from the interferon arm. The estimated 5- and 10-year relapse-free survival rates were 85% and 67%, respectively. The 5-year survival was 81%. Acknowledging that this was a crossover design, the survival outcomes were similar between the two groups [116]. The findings in this study have mirrored other long-term follow-up trials with pentostatin (Table 10.3).

Currently, pentostatin is given every 2 weeks usually at a dose of 4 mg/m^2 for 3–6 months until maximum response is achieved. Previous experience with high-dose pentostatin (twice the standard dose) was associated with serious infectious complications [118]. With this interrupted dosing schedule, febrile neutropenia is significantly reduced, especially in comparison to cladribine [63, 71]. Other common side effects of pentostatin include nausea, vomiting, photosensitivity, and keratoconjunctivitis [119].

Cladribine and pentostatin have amassed significant long-term data with many years of follow-up. Else et al. reported on outcomes of 233 patients with a median follow-up of 16 years. In this retrospective review, treatment with single cycle of cladribine or multiple cycles of pentostatin showed equal efficacy: complete remissions (76% vs. 82%) and overall survival (100% vs. 95%) [101]. Lopez et al. described a median treatment-free interval of 95 months with first-line pentostatin and 144 months with first-line cladribine; the difference was not statistically different (p = 0.476) [107]. Despite similar effectiveness, a single course of cladribine is generally considered the preferred first-line treatment because of its brief treatment duration and paucity of adverse effects.

Other Treatments

Splenectomy

Historically, splenectomy was the first effective therapy for HCL. Splenectomy did not affect bone marrow infiltration, but did remove a major site of hairy cell proliferation and alleviated the symptoms of hypersplenism [120]. Most studies noted 60–80% improvement of blood counts with rapid improvements in thrombocytopenia [121, 122]. These responses were not consistent. The degree of splenomegaly was not predictive of hematological improvement or duration of response [123] and most patients ultimately relapsed. The median time to failure with splenectomy was variable, ranging from 5.4 to 56.5 months [124]. No randomized trial has shown a survival benefit with splenectomy [121, 125]. The present indications for splenectomy are active and uncontrolled infection, the resolution of which can be rapid and reflects the improvement of peripheral blood counts. Splenectomy is used in the rare event of a splenic rupture and is beneficial in patients with splenomegaly and severe

Table 10.3 Long-term follow-up studies with pentostatin

Study	Patients (no.)	Patients (no.) with prior therapy	Median F/U (years)	Initial complete remission rate (%)	Relapse rate (%)	Overall survival
Cassileth et al. [117]	50	31	3.25	64	20	NA
Malosiel et al. [65]	238	154	5.3	79	15	89% at 5 years
Flinn et al. [116]	241	87	9.3	71	18	81% at 10 years
Else et al. [101]	188	108	16	82	44	95% at 15 years
Lopez et al. [107]	27	0	12.1	92	51	NA
Cornet [74]	99	0	4.8	82	23	NA

NA data not available

thrombocytopenia who are bleeding. Splenectomy can also be considered in the refractory setting as well as in the second trimester of pregnancy [121, 126].

Interferon

Interferon-alpha is an active agent in HCL and had a significant impact on treatment prior to purine nucleoside analogues. The exact mechanism of action is unknown, but it is thought that interferon-alpha acts as a cytostatic agent in HCL, inducing hairy cell differentiation and making these cells less responsive to growth stimuli [127].

In 1984, Quesada and colleagues first reported the successful use of partially purified alpha human interferon in seven patients with HCL. All seven had normalization of their blood counts with the responses maintained for 6–10 months [128]. Two recombinant interferon-alpha drugs were subsequently developed and approved by the FDA: interferon-alpha-2a and interferon-alpha-2b. Differing by only an amino acid, the recombinant forms showed equal efficacy [129].

Quesada's landmark trial has paved the way to many national and international trials. Treatment with interferon-alpha results mostly in partial remissions ranging from 69 to 87%. Few complete responses are noted and the duration of response is 18–25 months [129–131]. Long-term studies have reported improved survivals of 85–90% at 5 years [132, 133]. Interferon-alpha has activity even in previously treated patients and response rates are robust in patients with splenomegaly [131, 134]. The standard treatment for interferon-alpha-2b is 2×10^6 U/m^2 subcutaneously three times per week for 6–12 months and for interferon-alpha-2a is 3×10^6 U/m^2 three times per week for 12 months. The most common side effects of interferon-alpha are a flu-like syndrome consisting of fever, myalgias, and malaise. Rarely, central and peripheral nervous system complaints have been documented [128, 129].

Although interferon-alpha is an active agent in HCL, it does not induce the same complete responses seen with purine nucleoside analogues and thus is no longer utilized as initial therapy. Treatment with interferon-alpha should be reserved for patients with active infection who cannot receive a purine nucleoside analogue because of its associated immunosuppression.

Evaluation and Follow-Up of Treatment

Patients with HCL should be followed closely for months after treatment to evaluate for cytopenias, possible infectious complications, and ultimately treatment responses [113]. Recovery of blood counts may take weeks to several months following treatment with purine nucleoside analogues. In one study, the median recovery time to normalization of peripheral blood counts after the first cladribine course was 49 days (range 9–379 days) [71]. In addition to evaluating bone marrows for treatment response, translational studies have shown that soluble serum IL-2 secreted by hairy cells correlates closely with disease course and can be used as a noninvasive parameter for disease response [135]. Resolution of hepatosplenomegaly, adenopathy, cytopenias, and eradication of hairy cells from the peripheral blood and bone marrow by non-immunologic studies currently constitutes a complete response [136].

Despite robust responses to treatment with purine nucleoside analogues, long-term studies continue to show late relapses [76, 101]. With this in mind, researchers turned their attention to evaluating minimal residual disease (MRD) in posttreatment bone marrows, in the hopes of more completely eradicating hairy cell infiltrates. MRD can be identified by several techniques: immunohistochemistry using CD20, DBA.44, and CD45RO immunostains; immunophenotyping by flow cytometry; or polymerase chain reaction (PCR) [137–139]. Immunohistochemistry was initially thought to be more sensitive for detecting MRD [43]; however, upon direct comparison, PCR was found to be the most sensitive and specific test [138]. With increased diagnostic sensitivity, these studies have identified residual disease in 10–50% of patients previously thought to be in a complete remission [137, 140] (Table 10.4). This MRD usually represents <1% of the total cell population [137, 141].

Using a strategy of initial cladribine therapy followed by rituximab, researchers have shown that they can successfully eradicate MRD [144, 145]; however, it is not clear if this preemptive treatment strategy translates into improved clinical outcomes. Studies evaluating this question have shown mixed results. Sigal et al. reported on 19 patients who were

Table 10.4 Evaluation of minimal residual disease (MRD)

Study	Method of evaluation	Treatment	Patient (no.)	MRD (%)
Ellison et al. [137]	IHC with anti-CD20 and DBA.44	Cladribine	154	50
Hakimian et al. [140]	IHC with anti-CD20, anti-MB2, anti-UCHL-1	Cladribine	34	21
Wheaton et al. [141]	IHC with anti-CD20 DBA.44, anti-CD45RO	Cladribine	39	13
Matutes et al. [142]	IHC with anti-CD11c, anti-CD25, anti-CD103, and anti-HC2	Pentostatin	31	43
Filleul et al. [143]	PCR, IGH genes	Cladribine	10	100

in complete hematologic remission following initial treatment with cladribine with a median follow-up of 16 years [146]. Using flow cytometry and immunohistochemical staining (CD20, DBA.44, TRAP, and annexin positive), these investigators were able to determine that 47% of patients had no MRD. They also found that in patients with MRD or even gross bone marrow involvement, normal blood counts are possible. The study concluded that HCL is potentially curable and that patients with MRD can have long periods of complete remission [146]. More recently, Lopez et al. reported on a group of 82 patients initially treated with purine analogs and found a shorter treatment-free interval in patients with MRD compared to those without MRD (97 months vs. not reached, $p < 0.049$) [107]. Long-term follow-up with greater number of patients will be needed to fully appreciate the clinical significance of MRD.

Treatment for Relapsed and Resistant Disease

Purine nucleoside analogues elicit noteworthy responses in HCL. Despite this, there are a minority of patients who are refractory to treatment, and their prognosis is inferior. Researchers in Italy investigated biologic parameters in patients who did not respond to cladribine therapy. They found that the unmutated status of IGH variable region paralleled treatment failure and rapid progression of disease. Moreover, they identified defects in TP53 gene as a possible mechanism for resistance. These authors suggested that in such patients, a rituximab-based regimen may be more appropriate [147].

In addition to patients who are resistant to purine nucleoside therapy, long-term follow-up studies suggest that 20–40% of patients who initially had a response will eventually relapse. These patients have several options for therapy when treatment is indicated.

Re-treatment with Purine Nucleoside Analogue

Multiple studies have shown good efficacy when patients are re-treated with purine nucleosides. In the Scripps Clinic series, 62% of patients treated with a second course of cladribine on first relapse achieved a complete remission [71]. Similarly, in the Northwestern experience 83% of relapsed patients responded to a second cycle of cladribine therapy [106]. Even though cladribine and pentostatin have similar chemical structures, there is little clinical cross-resistance between the two drugs [148]. Else et al. showed that relapsed patients had a high rate of remission when treated with the other purine nucleoside analogue. On multivariate analysis, a shorter median duration of first remission was the only variable associated with a failure to attain a complete response [101]. Other studies have noted responses with purine nucleosides in the third- and fourth-line setting, but responses decline with each successive course [149]. No randomized trial exists to determine the optimal duration before re-treatment. Balancing efficacy with immunosuppressive effects of therapy, most experts recommend a 1-year interval before reconsidering purine nucleoside therapy [113].

Immunoconjugates and Targeted Therapies

Rituximab

Because hairy cells express CD20 brightly, rituximab, a chimeric monoclonal antibody against CD20, has become an important agent in salvage therapy. Hagberg first described rituximab as an effective therapy in a patient relapsing from HCL in 1999 [150]. Since then, multiple studies with rituximab have been conducted. Use of single-agent rituximab in the relapsed setting have shown response rates ranging from 25 to 80%, with complete response rates as high at 53% [151, 152]. Given these robust response rates, attention then turned to combination regimens with purine nucleoside analogs. Else and colleagues recently updated their results from a series of 26 patients treated with rituximab and either pentostatin ($n = 15$) or cladribine ($n = 11$) following purine analog treatment failure. Rituximab was administered at a dose of 375 mg/m^2 for 4–8 intravenous infusions (median 6.5). Twenty patients received rituximab concurrently and the remainder received sequential therapy. The overall response rate was 96%, and the complete response rate was 88%. At a median follow-up of 78 months, relapse-free survival following combination therapy was markedly improved compared to the RFS of the same patients following their prior first-line treatment (hazard ratio: 0.10; 95% CI: 0.03–0.32; log-rank $p < 0.0001$). Relapse-free survival at 10 years was 87% (95% CI: 75–100%) following combination therapy, versus 12% (95% CI: 0–24%) following the patient's same first-line treatment [153]. The authors did note certain discrepancies: some patients may have had a heavier baseline disease burden at the time of initial therapy compared to relapse, and 8 of the 26 patients received purine analog in combination therapy that they had not been previously exposed to. Though additional studies of rituximab combination therapy with purine analogs have also shown high response rates for classical [154–156] and variant HCL [157], true randomized trials are lacking.

BRAF Inhibitors

Building on the Italian group's discovery of the BRAF V600E mutation in a very high percentage of classical HCL patients, studies exploring the role of BRAF inhibitors have

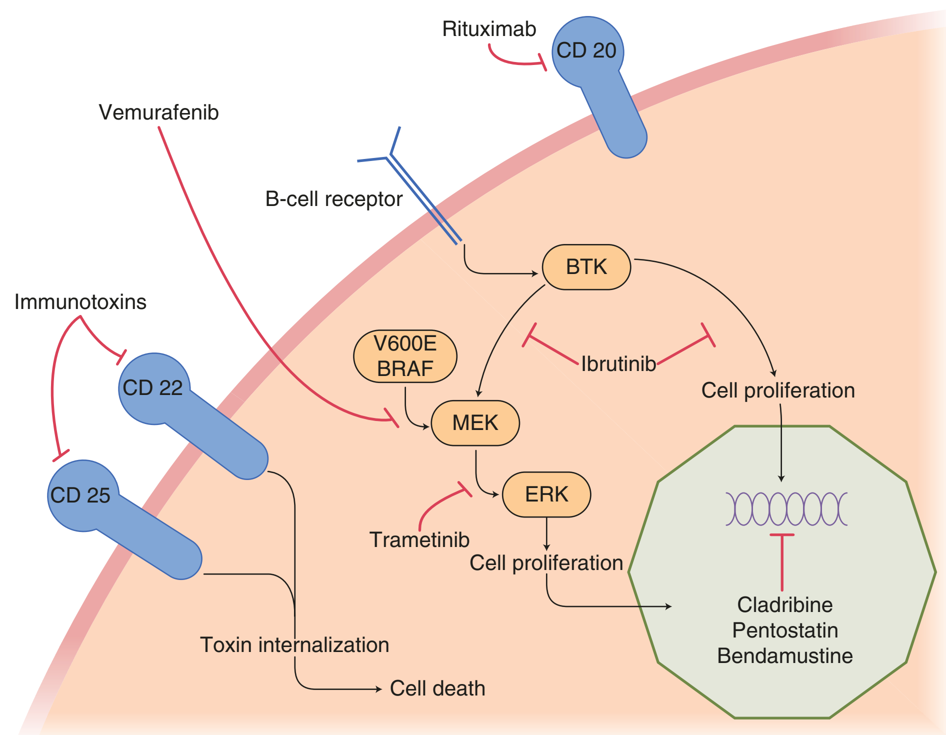

Fig. 10.5 Hairy Cell leukemia therapeutic pathways. *MEK* indicates mitogen-activated protein kinase, *ERK* extracellular signal-regulated kinase, *BTK* Bruton's tyrosine kinase

yielded encouraging results. Early case reports revealed rapid responses, including complete responses, in multiply treated relapsed and refractory classical HCL patients treated with the oral BRAF inhibitor, vemurafenib [158–161] (Fig. 10.5). More recently, results from two phase 2, single-group, multicenter studies of vemurafenib at a dose of 960 mg twice daily were published [162]. The studies were conducted in Italy and the United States. In the Italian study, treatment was administered for a minimum of 8 weeks and, if patients did not have a complete response, for a maximum of 16 weeks. At a median follow-up of 23 months, relapse-free survival was 19 months among patients with a CR. In the US study, patients received treatment on a continuous schedule for 12 weeks; however, patients with residual disease were allowed to receive vemurafenib for up to 12 additional weeks. Median treatment duration was 18 weeks and resulted in an overall survival rate of 91% after a median follow-up of 12 weeks. Therapy was generally well tolerated; drug-related adverse events were mostly grade 1 or 2. Toxicities included rash, arthralgias, and arthritis, among others. However, despite the high response rates, MRD was noted in all patients with complete responses at the end of treatment. In both trials, re-treatment with vemurafenib at relapse elicited some responses.

In the Italian study, bone marrow specimens from 13 of 26 patients were evaluated for phosphorylated ERK and PAX5 double immunostaining. Residual hairy cells expressing ERK were seen in 6 of the 13 patients. All 6 of these patients had a partial response to vemurafenib. Conversely, two patients with complete responses did not express phosphorylated ERK. Post hoc analysis revealed a prolonged median progression-free survival for patients with phosphorylated ERK compared to those lacking this (8 months vs. 13 months, respectively). In addition, residual disease (assessed by the Hairy Cell Index) was greater in patients with persistent phosphorylated ERK than those without measurable ERK. These investigators proposed that circumvention of BRAF inhibition may be explained by alternative mechanisms for reactivating MEK and ERK. The persistence of HCL cells and potential identification of resistance mechanisms indicate the need for additional therapy to improve response rates. One potential method may be to combine a BRAF inhibitor with another drug that targets the pathway, like an MEK inhibitor, as has been done in melanoma patients [163].

MEK Inhibition

The activity of the MEK inhibitor, trametinib, was recently supported by both in vivo and in vitro studies [164]. Pettirossi et al. isolated HCL cells from 26 patients and exposed them in vitro to active BRAF inhibitors (vemurafenib or dabrafenib) or trametinib. The in vitro results were subsequently validated in vivo in the phase 2 study of refractory and relapsed HCL patients treated with oral vemurafenib as detailed above. Vemurafenib, dabrafenib, and trametinib incubation resulted in dose-dependent MEK and ERK

dephosphorylation as well as considerable loss of the hairy morphology. Moreover, the ERK dephosphorylation and apoptosis appeared to be potentiated by the combination of BRAF and MEK blockade with dabrafenib and trametinib. These findings highlight feasible approaches for new treatment options and warrant investigation.

Recombinant Immunotoxins (CD22, CD25)

Recombinant immunotoxins are antibody-toxin chimeric proteins. By engineering the antibody moiety to target antigens expressed preferentially in HCL cells, the toxin moiety is able to exert lethal effects selectively. Recombinant immunotoxin research in HCL has focused on CD25 and CD22.

Kreitman et al. have extensively reported on the efficacy of BL22, a recombinant immunotoxin comprised of a pseudomonas exotoxin fused to a single-chain variable fragment of anti-CD22 [165–168]. Initial results revealed complete remissions in patients with HCL resistant to purine analog therapy [165]. Phase 2 testing of 36 patients with relapsed and refractory HCL, including three patients with variant HCL, was completed [167]. In this study, the complete response rate was 25% after one cycle. Twenty patients were then re-treated, and 47% achieved a complete response. Interestingly, response rates were higher in non-splenectomized patients without massive splenomegaly. Two patients experienced reversible grade 3 hemolytic uremic syndrome, which did not require plasmapheresis. Subsequently, moxetumomab pasudotox, a new recombinant immunotoxin with a 14-fold increased binding affinity for CD22, was developed. Phase 1 testing results are encouraging; the overall response rate was 86% and complete remissions were seen in 46% (13 patients). At 26 months, the median disease-free survival had not been reached. No dose-limiting toxicities were observed [169].

LMB-2 is a recombinant immunotoxin formed from the fusion of a CD25 antibody to the PE38 toxin. Phase 1 testing of this agent in 35 patients with chemotherapy-refractory CD25-expressing hematologic malignancies revealed measurable responses [170]. The most dramatic activity was seen in the HCL cohort. In this group of four patients, one had a complete response, two had partial responses, and the remaining patient had stable disease. Dose-limiting toxicities included reversible cardiomyopathy and transaminitis.

Alemtuzumab

Though it has not been extensively studied, there have been case reports suggesting the activity of alemtuzumab, a humanized monoclonal antibody against CD52, in HCL [171, 172]. Sasaki et al. treated a patient with variant HCL with splenic irradiation followed by alemtuzumab [171]. Splenomegaly resolved, and leukemic cells were eliminated from the peripheral blood by day 12. These reports warrant further investigation into the role of CD52 targeting in the treatment of HCL.

Bendamustine

Bendamustine is a chemotherapeutic agent with features of both alkylators and purine analogs. Following its initial report [173], bendamustine was further studied in relapsed and refractory HCL patients [174]. The results were encouraging; the overall response rate was 100%. At a dose of 90 mg/m^2 on days 1 and 2, for 6 cycles at 4-week intervals, 67% achieved CR, 100% of which were without MRD. At a median follow-up of 31 months, all complete responders with absent MRD remained in CR. Phase 2 trials are currently under way [175].

Ibrutinib

Ibrutinib, a selective and irreversible inhibitor of Bruton's tyrosine kinase (BTK), has activity in multiple low-grade lymphoproliferative disorders including chronic lymphocytic leukemia, mantle cell lymphoma, and Waldenstrom's macroglobulinemia. Recently, Sivina et al. reported their findings showing that BTK protein is expressed in HCL cells, and ibrutinib significantly inhibited HCL cell proliferation, cycling, and survival [176]. Preliminary safety and efficacy data was recently presented [177], and a phase 2 clinical trial is currently under way [178].

Conclusion

Since its initial description more than 50 years ago, the natural history of HCL has been dramatically altered. In the era of Bouroncle, treatment options were few, including splenectomy, and the median survival was only 4 years. Now with purine nucleoside therapies, many patients enjoy long-term remissions frequently surpassing 10 years with good quality of life. The final chapter in this remarkable tale, however, has not been written. Despite excellent responses to the purine nucleoside analogues, cladribine and pentostatin, a small minority of patients will not respond and a proportion who do will eventually relapse. Though these patients respond upon re-treatment, the responses are less rigorous and there is a concern that further therapy will cause long-term immunosuppression. Moreover, disease-free survival curves have failed to show a plateau after 10 years [149]. A few long-term studies, however, have suggested that some patients may be cured. Focusing attention on identifying refractory

patients with HCL through biologic parameters and employing treatment strategies with targeted agents, monoclonal antibodies, and recombinant immunotoxins are currently under investigation. Introduction of BRAF targeting agents represents a major therapeutic advance and addition to the therapeutic armamentarium for patients with refractory or relapsed disease. More research is still needed in understanding the fundamental biology of this disease. Filling in the gaps in our knowledge of the pathophysiology will aid in the development of better treatments with the prospect of more patients enjoying long-term disease-free survival and perhaps even a curative strategy. Also, delving into the biology of HCL will provide insights and potential treatment directions for more common indolent lymphoproliferative diseases.

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Part II

Acute Leukemias

History of Acute Leukemia

11

Emil J Freireich

Introduction

In 1953, the United States Public Health Service opened the 500-bed clinical center on the Bethesda campus of the National Institutes of Health. James Holland, who had trained at the Francis Delafield Hospital of the Columbia University School of Medicine, was among the first clinicians to join the faculty of the National Cancer Institute's clinical center and he began to utilize these agents for the treatment of children with acute leukemia. In 1954, Charles Gordon Zubrod was recruited to the clinical center to become the head of the medicine branch in the Cancer Institute and he recruited Emil Frei III, who was his chief resident, to come with him to the Cancer Institute. Early in 1955, Emil J. Freireich joined the Cancer Institute with a background in hematology.

Dr. Holland left the Cancer Institute in 1954 to move to Buffalo, the Roswell Park Memorial Institute, and when Freireich arrived in Bethesda there were three patients in the hospital with acute lymphoblastic leukemia. What the new group decided to do was to utilize the newly described technique of the prospective randomized controlled clinical trial which was so successful in developing the antimalarial drugs during the war, and secondly, to test the hypothesis that combination chemotherapy would provide synergistic effects—this had been demonstrated in the treatment of infectious diseases, particularly tuberculosis. In reviewing what was published up to that date on the chemotherapy of acute leukemia, these young investigators discovered that the agents that were described to induce temporary remissions when used singularly [1–8] did not significantly affect the natural history of acute leukemia [9]. They initiated a collaborative study between the group at the Cancer Institute in Bethesda and Dr. Holland and his group at Roswell Park in Buffalo. The goal of the first study was to evaluate the effectiveness of a combination of 6-mercaptopurine and methotrexate. They first reviewed the world's literature on the normal values for blood and bone marrow, physical findings, and symptoms, and published objective criteria for defining complete remission, partial remission, and hematological improvement which were to be used in this cooperative group study [10].

Secondly, a detailed research protocol was constructed and agreed to by the investigators at both institutions, and they devised data collecting flow sheets which mandated the measurement of bone marrow, blood, physical findings, and symptoms at regular intervals. This first study was a landmark study in that it established the feasibility of doing formal clinical trials, and it provided objective quantitative information of the frequency and duration of response and survival for a group of 63 consecutive patients [11].

Because the results of the combination study looked promising, the second protocol, Protocol 2, systematically compared the combination to each of the two drugs used as single agents followed, on failure, by a crossover to the other agent. These studies were designed based on mouse leukemia studies conducted by Lloyd Law and Abe Golden which suggested not only the concept of combination chemotherapy, but also the possibility that there was collateral sensitivity, i.e., the disease resistant to one agent would have enhanced sensitivity to the other. Protocol 2 had three arms—the combination of methotrexate and 6-mercaptopurine which was given at 60% of their maximum tolerated dose that was worked out in mouse toxicology studies because these two drugs were both myelosuppressive agents; and 60% of each combined to a maximum tolerated dose for the two drugs, which was compared with the full therapeutic dose of methotrexate, followed by 6-mercaptopurine upon failure; and the third arm was 6-mercaptopurine followed by methotrexate upon failure. While this study was being prepared, the results of Protocol 1 were publicized through scientific meetings and a number of institutions, specifically ten institutions, elected to join in the study; so Protocol 2 was

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able to accrue a much larger sample size in a reasonable period of time. The results of that study were positive in the sense that the combination of the two agents was more effective—in terms of frequency and duration of response, and survival—than the two agents used in either sequence, but no collateral sensitivity was demonstrated [12].

It was clear that one strategy for the control of acute leukemia was to discover new agents with unique mechanisms of action. In the first such study undertaken by the National Cancer Institute group—the study of 6-azauracil which was shown by Arnold Welch in experimental animals to have a high degree of activity—the results of the Phase 2 study revealed some hematological improvements in the patients, suggesting that this was a potential drug for using in newly diagnosed patients [13]. But, before that was undertaken, they recognized that they had little information on patients who had been extensively treated previously with the three active drugs. So, they undertook the first prospective randomized controlled trial in cancer research comparing 6-azauracil to a placebo in these terminal advanced refractory leukemia and found that there was no significant difference between those treated with placebo or the active drug [14]. This was an important study because it demonstrated the importance of concurrent controls and quantitative assessment of response—because the disease was not inevitably progressive and quantitative information to build new hypotheses was essential.

For Protocol 3 [15], they hypothesized that the patients would be in better physical and clinical condition in remission, and therefore, they would have a better opportunity to assess the activity of new agent. The clinical trial was designed to expose all children to the most active remission-inducing drug which, at that time, was hydrocortisone, resulting in approximately 60% of the children achieving complete clinical and hematological remission. The children were then randomized to receive either placebo or 6-mercaptopurine, which was known to be active in inducing complete clinical and hematological remission in approximately 30% of the patients with active leukemia. The study was designed in an innovative way using a sequential design, so that when the probability that one was better than the other was greater than 95% the study stopped automatically. The results of this study were impressive because it first demonstrated a substantial prolongation of remission with 6-mercaptopurine, and it established that corticosteroid-induced remissions had a median remission of only 8 weeks and all patients had recurrence at approximately 10 months. In contrast, the patients who received mercaptopurine, all patients, not just 30%, had significant prolongation of remission and approximately 10–15% of the patients remained in remission for more than a year, which was an unheard of result with any prior therapy. Moreover, those who received placebo and were subsequently treated with 6-mercaptopurine and methotrexate had overall survival which was comparable to the patients who received 6MP during remission, indicating that their survival was not significantly compromised. The results of this study demonstrated, for the first time, the principle of adjuvant chemotherapy, i.e., chemotherapy given to patients free of clinical disease was substantially more effective than the same therapy given to patients with active disease. That was the major outcome of this study, even though that was not the initial plan.

The next major development was the discovery of the vinca alkaloids by Irving Johnson working at Eli Lilly Laboratories. In an effort to develop new antidiabetic drugs, Eli Lilly screened many natural products and the extract from the periwinkle plant contained alkaloids that had little effect on glucose metabolism, but did cause substantial myelosuppression in mice. Dr. Johnson had the impressive thought that this drug might be active in leukemia. To that end, Eli Lilly, at great expense, extracted this alkaloid from very large quantities of the periwinkle plant and initiated clinical trials in Children's Hospital in Boston and with the group at the National Cancer Institute. The Phase 2 trial with this drug gave dramatic responses. Over 50% of the children with far advanced disease refractory to the three known agents developed rapid, complete clinical and hematological remissions [16]. Like steroids, these remissions were short, approximately 8 weeks on average, but the results were impressive and the limiting dose toxicity for this drug was not myelosuppression, but central nervous system and peripheral nervous system toxicity. The group had learned that one could add the full therapeutic dose of adrenocortical steroids, which is not myelosuppressive, to the myelosuppressive drugs methotrexate or 6-mercaptopurine, and in both instances, frequency of complete response to the combination was higher than that predicted from the results of each individual drug used in previously untreated patients. This led the NCI group to consider the possibility of combining all four known active agents into a single therapy because they recognized that vincristine and prednisone did not have additive toxicity, nor were they myelosuppressive. They knew the maximum tolerated dose of the combination mercaptopurine methotrexate, so they created the first multi-agent four-drug chemotherapy regimen, and even more importantly created the first eponym for this combination (VAMP): vincristine, amethopterin, mercaptopurine, and prednisone. The results of the VAMP study were impressive—over 90% of the children entered remission rapidly and with minimal side effects. Since they had recognized the importance of adjuvant chemotherapy from Protocol 3, the NCI investigators used early intensification of the remission induced by VAMP and gave three induction courses to the children in remission. What they observed was that the duration of remission was as long as that achieved with adjuvant 6-mercaptopurine. Then they introduced the concept of continuous intermittent re-induction therapy specifically; they gave the same four agents for induction, consolidation, and continued intermittent therapy at monthly intervals for 1 year (POMP). By 1964, with over 3 years of follow-up, they reported

that one-third of the patients were still alive and disease free, and they suggested that these patients were "cured" of their childhood lymphoblastic leukemia—a prediction which has proven to be correct [17–19].

Treatment of childhood ALL progressively improved over the next 40 years with the addition of new agents, such as asparaginase. The recognition of the importance of meningeal leukemia and the role of prophylaxis for central nervous system involvement is such that at present at least 80–90% of such patients are literally cured of their disease with chemotherapy alone [20].

When the activity of 6-mercaptopurine, methotrexate, and prednisone was described, it was early recognized that the cytological details of the morphology of these leukemia cells were suddenly extremely important. Those leukemia patients who had a lymphoid phenotype responded dramatically, whereas those who had myeloid characteristics had the same natural history as in the untreated state and were found to be quite resistant to chemotherapy [21]. The other prognostic factor that was identified was that the optimal responses occurred in patients under 10, and in patients over 20, the frequency of response and survival was significantly lower.

In addition, they recognized the importance of the degree of leukocytosis as an important prognostic factor.

VAMP was primarily active in children with acute lymphoblastic leukemia. The investigators attempted the same four-drug combination (POMP) therapy in children with myeloid phenotype and in young adults. Although the effects were much less dramatic and quantitatively less effective, they nonetheless reported that approximately 25% of adults in this case (median age well below median age of approximately 40) could achieve complete hematological remission, and with intermittent maintenance, these remissions could last for approximately 1 year. These data were assembled and submitted to the journal, *Blood*, for publication. The manuscript was rejected and Dr. Dameshek, founder of the journal and the editor-in-chief, was inclined to write editorials on the progress in hematology and he wrote an editorial in 1965 which said, "However, not only was this method unscientific, but the initial toxic reaction may be lethal, particularly in adults. Furthermore, it has not yet been clearly shown that this treatment program offers a significant advantage over more conservative approaches. In general, however, they may be thought to represent 'gropings' which engender little enthusiasm for long-term advantages" [22].

The situation for adults in 1965 was still poor—prednisone and 6MP gave 10% responses, and POMP reported to give 25% responses; these were largely ignored by the medical community as being too toxic for adult patients. The breakthrough occurred with the description of the activity of arabinosyl cytosine in 1963 [23].

In 1968, Ellison and Holland described the induction of remission in adults with acute myeloblastic leukemia [24]. Based on the animal experiments which were conducted by Skipper and Schabel at Southern Research Institute [25], it was demonstrated that Ara-C was schedule dependent.

M.D. Anderson reported that a group of patients in 1967 were treated by continuous infusion over 120 h or 5 days [26]. The Southwest Oncology Group conducted a dose schedule study which demonstrated that single injection of Ara-C as high as 3 g/m^2 did not cause myelosuppression, but infusion of 48 h or longer had dose-responsive myelosuppression [27]. The Southwest Oncology Group then compared 48- and 120-h infusions which showed higher frequency and longer duration of response with 120-h infusions [28].

In 1964, an Italian group discovered the natural product, daunorubicin, an anthracycline, antibiotic [29]. And in 1969, adriamycin, another anthracycline antibiotic, was discovered [30]. Both of these drugs had substantial single-agent activity in patients refractory to Ara-C. What followed was a series of studies combining the anthracycline antibiotics with Ara-C in its best schedule. Many of these combinations were studied in cooperative groups, but the one that proved to be best tolerated and most effective for the highest number of patients was the famous 3 and 7 regimen—3 days of daunorubicin and 7 days of continuous infusion of arabinosyl cytosine [31].

The academic hematology community received these reports with skepticism. As late as 1974, one of the giants of hematology, William Crosby, published "chemotherapy should be used only sparing in related cases of leukemia, since the treatment may be killing more patients than proponents of aggressive therapy realize" [32].

In 1976, the editors of the Archives of Internal Medicine published a series of papers in rebuttal of Crosby's comments supporting the use of chemotherapy for adults with AML [33–35].

In 1981, it was clear that the natural history of AML had changed substantially at least for a fraction of these patients [36]. For the first time, completed 5-year survival was observed in approximately 15% of the patients diagnosed with AML before 1976. Shortly thereafter, a major review of the treatment of acute leukemia in many centers and in the cooperative groups was conducted and it was clear that these results from a single institution were confirmed not only in many institutions but also in multi-institution studies. And an analysis of long-term survival was published in 1983 [37].

With the advent of techniques to study the human chromosomes, including banding to identify the individual chromosomes, many investigators reported that acute leukemia patients had a number of abnormalities in the chromosomes which were apparently random. However, in 1979 Trujillo recognized the first nonrandom chromosome abnormality—the translocation between chromosomes 8 and 21 [38]. Over the following years, many nonrandom chromosome abnormalities were identified which allowed a systematic classification of acute leukemia into groups [39]. Favorable was the

inversion 16, the translocation 8:21, and the translocation 15:17, which constituted approximately 20% of all the acute myeloid leukemias. For these diseases, chemotherapy provided long-term (3 years or longer) survival for the majority of the patients. About one-third of the patients had complex cytogenetic and these were clearly unfavorable, where long-term survival occurred rarely. Then the remaining 50% were intermediate: mostly diploid patients who had an intermediate outcome of approximately 20% long-term survival. The most dramatically distinctive group of AML patients was the group which had the translocation between chromosomes 15 and 17 which created a neo-gene (the RARA/PML gene) which was susceptible to remission induction by all-trans-retinoic acid. With the addition of arsenic trioxide it became possible for this small subset of AML patients (approximately 6–10%) to enter complete remission with long-term survival and without any cytotoxic chemotherapy [40]. The discovery of the cytogenetic classifications of patients formed the basis for the new molecular oncology so that now specific genetic traits are used not only for diagnosis and choice of therapy, but also for the detection of residual disease and for guiding the courses of therapy [41].

Another important advance was the development of myeloablative allogeneic stem cell transplantations [42, 43]. This was shown to result in long-term survival for a significant fraction of patients with acute leukemia. This therapy has become a significant therapy, particularly for patients in remission. The transplant techniques have also improved rapidly with the use of peripheral blood stem cells, reduced intensity conditioning and improved tissue typing, matched unrelated donor pools, and improved control of complications such as infection and graft-versus-host diseases [44].

Conclusion

Considering the transformation of AML from a universally fatal untreatable disease in the mid-1960s to one with a significant portion of cured patients and a very high proportion of patients who respond to therapy in a half century, this rapid progress suggests strongly that knowledge about the biology and therapy of acute leukemia will increase at an accelerating pace and hopefully lead to control of this disease in the near future. Recent authoritative reviews of progress in acute leukemia treatment have appeared [45, 46].

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The Etiology of Acute Leukemia

12

J.N. Nichol, M. Kinal, and W.H. Miller Jr.

Introduction

Leukemia is a clonal disorder of deranged and disordered hematopoiesis that results from the acquisition of mutations in hematopoietic progenitors that confer a proliferative and/or survival advantage, and impair hematopoietic differentiation. In the case of acute myeloid leukemia (AML), these progenitors are from the myeloid lineage and for acute lymphocytic leukemia (ALL), these progenitors are from the lymphoid lineage (Fig. 12.1). Specifics concerning the clinical features and treatment of acute leukemia can be found elsewhere in this textbook. This chapter focuses on the molecular pathogenesis of acute leukemia.

Over a decade ago, Kelly and Gilliland [1] proposed a two-hit model for the development of AML, which is also applicable to the development of acute lymphoblastic leukemia ALL, whereby they hypothesize that these diseases emerge as a consequence of an association between at least two broad classes of mutations (Fig. 12.2). Class I, or activating, mutations typically result in the aberrant activation of signal transduction pathways and provide a proliferative and/or survival advantage to hematopoietic progenitors. Class II mutations arrest differentiation because of loss-of-function (LOF) mutations in key transcription factors or cofactors that are important for normal hematopoietic differentiation.

Cumulative evidence from different murine models of leukemia provides support for the necessity of cooperative transforming events for leukemogenesis. Induced mutations in these models result in increased self-renewal capacity and reduced differentiation, but are not sufficient to induce malignant transformation, and acute leukemia was not observed in mice until the mutations were combined with a dose of the chemical mutagenic agent, *N*-ethyl-*N*-nitrourea (ENU) [2–4].

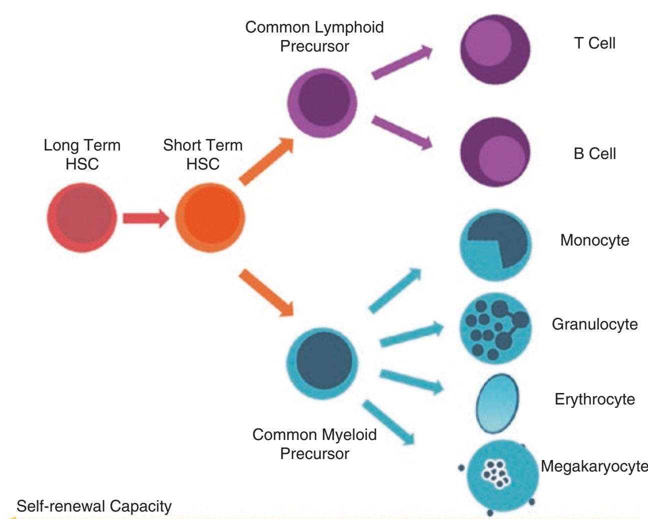

Fig. 12.1 *The hierarchy of hematopoietic development*. Long-term stem cells (HSCs) develop into different precursor cells, whose self-renewal capacity and developmental potential become progressively more restricted

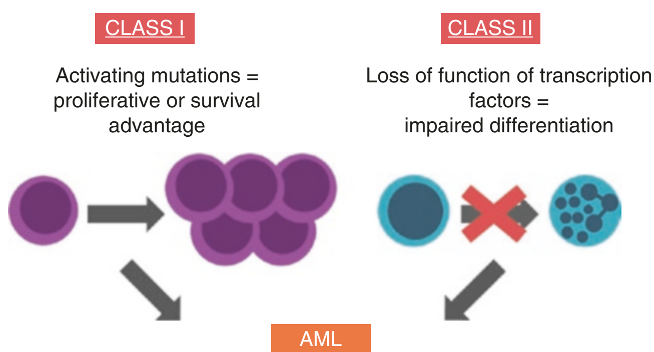

Fig. 12.2 *The two-hit model of acute leukemogenesis*. This model hypothesizes that AML and ALL are the consequence of a combination of at least two broad classes of mutations. Class I mutations involve an activating lesion in signaling pathways and confer a proliferative and/or survival advantage to hematopoietic cells. Class II mutations lead to an arrest of lymphoid or myeloid differentiation as a result of loss of function of transcription factors or cofactors that are important for normal hematopoietic differentiation

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A study of monozygotic twins who both developed ETV6/RUNX1-positive ALL [5] with identical chromosome breakpoints, but with different timelines, provides further support for the requirement of secondary genetic insults. Additionally, common leukemia- and lymphoma-associated genetic rearrangements are found in the peripheral blood of individuals with no history of either disease [6] and there are rare familial leukemia syndromes, involving germline *CEBPA* [7] and *RUNX1* mutations [8], where affected individuals exhibit an enhanced, but not guaranteed, risk of developing leukemia.

Over the past decade, technological advances have revolutionized our ability to interrogate cancer genomes, culminating in whole-genome sequencing (WGS), which provides genome-wide coverage at a single base-pair resolution. The information provided by these more sophisticated methods reveals that class I and II mutations are only one part of a more intricate framework. To date, the tumor genome has been sequenced in hundreds of cases of acute leukemia [9–14], and the latest numbers suggest that each AML genome contains hundreds of mutations, including anywhere between 5 and 23 coding mutations [14]. Most of these mutations are “background,” or passenger mutations that were acquired during normal aging of hematopoietic stem cells, but some novel “driver” mutations have been discovered through WGS analysis [11]. Many of these mutations do not fit into either Class I or Class II, suggesting that the “two-hit model” is perhaps oversimplified [9]. Moreover, there is evidence that the sequence and timing of the aberrations during development may be important, suggesting that the cellular milieu in which the transcripts are expressed is relevant. This has been reported, for example, in acute promyelocytic leukemia (APL). The PML/RARA fusion protein in APL may occur at any point in the development of the myeloid cell, but is associated only with leukemia if the translocation occurs at an early stage, while expression of the fusion in late myeloid cells has little effect [15].

Based on the discoveries made by next-generation sequencing, we can now expand the initial two categories of genes relevant for leukemic pathogenesis into nine [9] (Fig. 12.3). In this review, we discuss in detail some of these nine categories and, in particular, we highlight the recent advances in the understanding of acute leukemia in the WGS era.

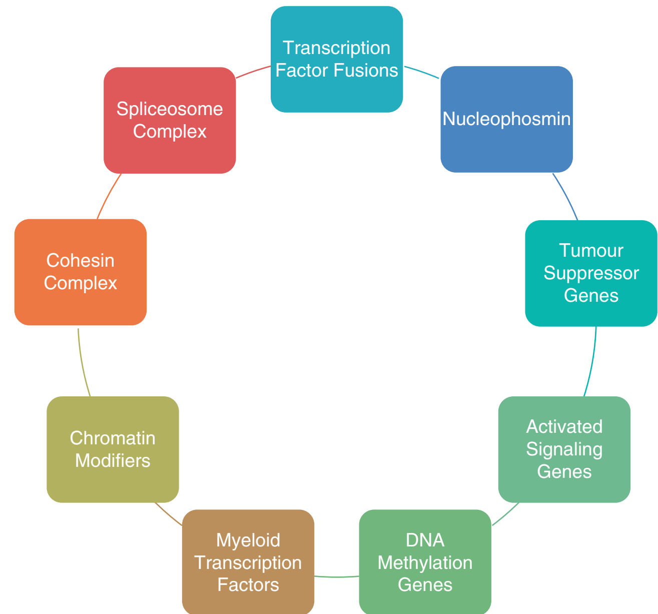

Fig. 12.3 *Functional categories of gene mutations in acute leukemia*. Genes found to be mutated via whole-genome sequencing of patient samples can be organized into nine categories based on related biological function

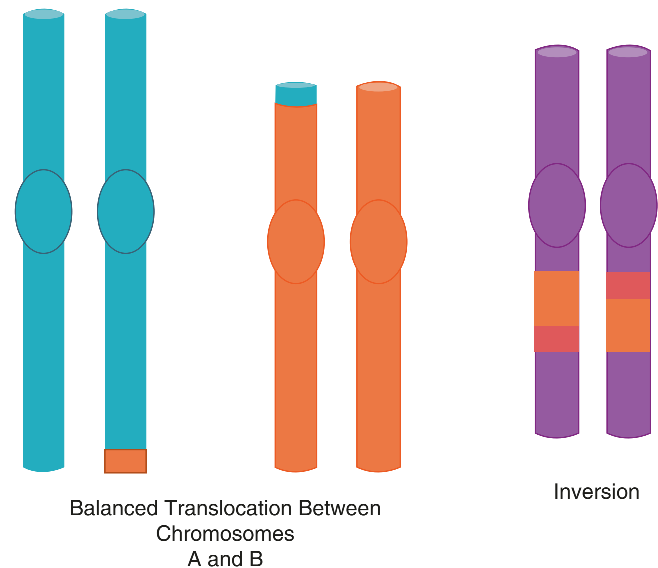

Fig. 12.4 *Structural chromosomal abnormalities*. A translocation arises when an exchange of chromosomal material takes place between two different chromosomes. If the translocation is balanced, the pieces of the chromosomes are rearranged but there is no loss or gain of genetic material (*left*). An inversion occurs when a segment of the chromosome is excised, rotated 180 degrees, and then reinserted into the same chromosome (*right*)

Transcription Factor Fusions

There is a long-established relationship between structural genomic aberrations, especially chromosome translocations and inversions, and acute leukemia. Specific cytogenetic abnormalities are often uniquely associated with clinically distinct subsets of the disease. Identifying the DNA sequences surrounding the chromosomal breakpoints has had an enormous impact on our understanding of leukemogenesis, carcinogenesis in general, and normal cellular functions of the protein products of the involved genes.

A chromosome translocation is a structural abnormality resulting from the exchange of pieces between two nonhomologous chromosomes. Reciprocal translocations are those in which there is no obvious overall loss of chromosomal material, and they probably result from a failure to properly repair DNA double-strand breaks (Fig. 12.4).

Several recurrent reciprocal translocations are frequently found in acute leukemia. Specific translocations are often consistently associated with specific subtypes of leukemia, providing strong support that they represent causal events during leukemogenesis in developing hematopoietic cells. For example, the t(15;17) is found only in patients with APL and not other forms of leukemia, while the t(1;19) is found only in the leukemic cells of patients with B-cell precursor acute lymphoblastic leukemia (ALL). However, there are exceptions to this phenomenon. For instance, the t(4;11) translocation occurs in patients with both AML and ALL. Chromosomal inversions are also seen in acute leukemia. An inversion occurs when a single chromosome undergoes breakage and rearrangement within itself (Fig. 12.4).

Identifying the genes disrupted by breakpoints in translocations and inversions provided the first mechanistic insights into understanding why chromosome abnormalities can be leukemogenic.

X/RARA

Acute promyelocytic leukemia (APL) is a subtype of acute myelogenous leukemia, representing 5–8% of AML cases in adults. At the genetic level, APL is characterized by a specific chromosomal rearrangement between the retinoic acid receptor alpha (*RARA*) on chromosome 17, and a number of partners. The majority of patients (98%) present with the 15;17 translocation, t(15;17), which results in a fusion of *RARA* with the promyelocytic leukemia (*PML*) gene on chromosome 15 [16, 17]. In normal cells, the retinoic acid receptors (RARs) heterodimerize with the retinoid X receptors (RXRs) to form a transcriptional complex. In the absence of ligand, all-trans retinoic acid (RA), a vitamin A derivative, the heterodimers are found bound, along with inhibitory co-repressor molecules, to the retinoic acid response elements (RAREs) of target genes. Treatment with ligand causes a release of the co-repressors and a concurrent recruitment of co-activators, leading to initiation of transcription of genes important for stimulating myeloid differentiation and regulating the cell cycle.

In APL cells, the prevailing view is that the leukemic effects of the resulting chimeric protein, X/RARA, are due to its function as a dominant negative inhibitor of normal retinoid receptor function. The chimera locates to promoters normally regulated by RARA, aberrantly recruits co-repressor proteins, and thereby inhibits the RARA-mediated gene expression. Therapeutic doses of RA can circumvent the differentiation block [18].

To date, eight different chromosomal translocation partners have been identified in patients with APL [19–26] (Fig. 12.5), and, as mentioned above, all involve the *RARA* gene on chromosome 17q21. The N-terminal fractions contributed by the fusion partners donate additional dimerization domains. These acquired dimerization domains, together with the DNA-binding domain of RARA, are required for the oncogenic effect of the fusion proteins and promote formation of chimeric receptor homodimers and provide additional co-repressor binding domains. The resulting X/RARA fusion proteins have been shown to recruit the HDAC, NCOR1 and NCOR2 (NCoR) complex, DNMT1, DNMT3A, repressive histone methyl-transferases, and polycomb group proteins.

However, disruption of the normal RARA signaling pathway does not solely account for the pathogenesis of APL. As mentioned previously, the differentiation block mediated by the X/RARAs is necessary, but not sufficient to cause APL. This suggests that a second transformative event is required for full neoplastic development. Mutations in oncogenes such as *FLT3* can cooperate with the fusions to provide the second hit necessary to generate leukemia. The presence of these second mutations might also explain why APL cannot be cured by differentiation therapy with ATRA alone, but is highly curable by combinations of ATRA and cytotoxic chemotherapy. Additionally, some translocations generate the reciprocal *RARA/X* fusion genes which can result in the co-expression of their transcripts in leukemic blasts [27–29]. Important roles in oncogenesis are now being identified for the co-expressed RARA/X reciprocal fusion proteins [27, 28, 30, 31]. Additionally, the partner proteins in APL have important growth-regulatory roles in normal myeloid cells. It may

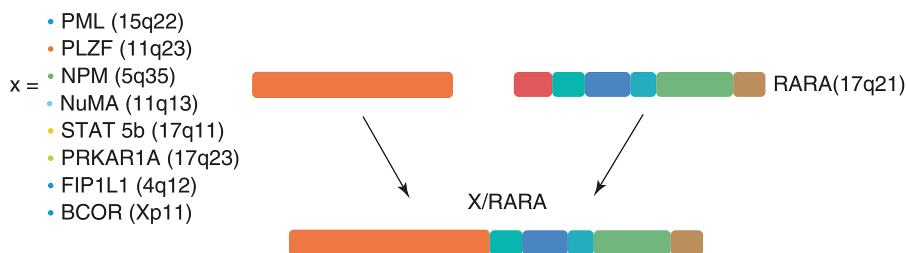

Fig. 12.5 *Identified chromosomal translocations associated with APL.* Acute promyelocytic leukemia (APL) is the result of chromosomal rearrangements in which part of the retinoic acid receptor alpha (*RARA*) gene is linked to the amino-terminal domains of eight genes (*X*) that encode different nuclear proteins. All of the translocations produce a chimeric protein (X/RARA) that disrupts the normal function of RARA and arrests the maturation of myeloid cells at the promyelocyte stage. The most common translocation, t(15;17), involves a break in chromosome 15 and disrupts the promyelocytic leukemia (*PML*) gene, producing the *PML/RARA* fusion

be that the loss of one allele of the partner protein combined with the effects of the X/RARA or reciprocal RARA/X fusion protein, which compromises normal partner protein function, contributes to the development of leukemia.

Core Binding Factor Rearrangements

Core binding factor (CBF) leukemias refer to a subset of AML bearing t(8;21)(q22;q22), which generates the RUNX1/CBFA2T1 fusion protein (previously known as AML1/ETO) and inv.(16)(p13q22), which generates the CBFB/MYH11 fusion protein. These genomic rearrangements are characterized by disruption of the *RUNX1* gene at 21q22 and the *CBFbeta (CBFB)* gene at 16q22, respectively. These are two of the most prevalent cytogenetic subtypes of AML.

Native RUNX1 and CBFB form a heterodimeric transcription factor [32] which regulates the transcription of genes required for the development of definitive hematopoiesis, including macrophage colony-stimulating factor (CSF) receptor [33], granulocyte-macrophage CSF [34], myeloperoxidase [35], and interleukin-3 [36].

RUNX1/CBFA2T1

The chromosomal translocation t(8;21) fuses the DNA-binding runt domain from RUNX1 (previously known as AML1) to almost the entirety of the CBFA2T1 (previously known as ETO) repressor protein [37]. The t(8;21)(q22;q22) translocation is present in 10–15% of AML cases and is associated with the M2 subtype of AML. Expression of the resulting fusion protein, RUNX1/CBFA2T1, in mouse models induces a pre-leukemic state, but requires cooperating mutations for full leukemogenesis [38–41]. An early hypothesis for the pathogenic role of RUNX1/CBFA2T1, postulating that it functions as a transcriptional repressor, was dervied from models based on the archetypal leukemiaassociated transcription factor, PML/RARA, and are supposrted by several lines of evidence:

1. Repression by RUNX1/CBFA2T1 is mediated by the CBFA2T1 moiety, which recruits co-repressors, such as HDACs 1–3, NCoR, SMRT, and mSin3A [42–44], and produces the secondary effect of altering the chromatin structure of target promoters.
2. RUNX1/CBFA2T1 binds to the promoters of tumor-suppressor genes which are normally activated by RUNX1, such as p14^{ARF} [45] and CCAAT/enhancer-binding protein alpha (C/EBPA) [46], and represses their transcriptional activation.
3. Treatment of t(8;21)-positive AML cell lines with HDACis [47] and DNMT inhibitors relieves the repressive effects of RUNX1/CBFA2T1 on chromatin, gene expression, and cell differentiation [48].

However, evidence has suggested that the model of RUNX1/CBFA2T1's leukemogenic effect through repressive transcriptional activity needs to be broadened. Microarray data shows that RUNX1/CBFA2T1 activates as many genes as it represses, including genes not normally under the control of wild-type RUNX1. Many of the genes upregulated by RUNX1/CBFA2T1, for example *JAG1* [49], play a role in promoting stem cell renewal. Furthermore, RUNX1/CBFA2T1 is a potent transcriptional activator of the anti-apoptotic *BCL2* gene [50]. Thus RUNX1/CBFA2T1 expression may result in leukemia by two opposing effects on transcription: by simultaneously repressing tumor-suppressor genes while upregulating genes that drive expansion or prolong the survival of t(8;21) early multipotent progenitors.

Furthermore, researchers have identified, in patients, a spliced isoform of the RUNX1/CBFA2T1 fusion protein, called AE9a, which is coexpressed with the full-length fusion and is capable of inducing leukemia in a murine transduction/transplantation model [51]. High levels of AE9a in patients are associated with poor disease outcome [52] and it was recently demonstrated that binding of AE9a to CBFB, leading to dysregulation of Notch target genes, is necessary for AML initiation [53].

Finally, an alternative explanation for the pathogenesis of t(8,21) acute myeloid leukemia is the loss of function of lineage-program-transcription factors [54]. These transcription factors strictly control gene expression during hematopoiesis, and instruct a precursor cell to commit to a certain differentiation program by initiating expression of a characteristic set of lineage-specific target genes in response to diverse signals [55]. There are four central hematopoietic transcription factors that are functionally repressed by RUNX1/CBFA2T1 through protein-protein interactions: PU.1 [56], CEBPA [57], GATA-1 [58], and the E proteins [59]. These inhibitory interactions with RUNX1/CBF2AT1 may expand stem cell pools by promoting stem cell renewal and blocking commitment to the various lineages. The expanded stem cell pool may then be primed for leukemic transformation through acquisition of additional mutations. The development of these secondary mutations is again facilitated by RUNX1/CBFA2T1 expression through the fusion protein's ability to repress DNA repair genes [49].

CBFB/MYH11

Inversion of chromosome 16, inv.(16), or the related t(16;16) is associated with the M4Eo subtype of AML, and results in the fusion of *CBFB* with *MYH11*, the gene encoding smooth-muscle myosin heavy chain. The resulting fusion protein CBFB/MYH11 is composed of the heterodimerization domain of CBFB fused to the C-terminal coiled-coil domain from MYH11. Studies suggest that CBFB/MYH11 has a higher affinity for RUNX1 than wild-type CBFB [60], and at the molecular level, CBFB/MYH11 has been suggested to alter

the normal transcription program by several mechanisms. These include the tethering of RUNX1 outside the nucleus [61, 62], recruitment of HDACs/co-repressors [63, 64], and inhibiting RUNX1 activity [65]. However, most of these studies were based on in vitro and overexpression experiments.

Furthermore, there are data suggesting that CBFB/MYH11 has activities independent of RUNX1/CBFB repression. CBFB/MYH11 expression has been shown to reduce levels of CEBPA protein, which is crucial for normal granulopoiesis, without affecting mRNA levels, but rather by the translational inhibition of CEBPA [66]. Additionally, microarray analyses have determined that CBFB/MYH11 expression results in a significant upregulation of many genes, including those involved in DNA replication, cell cycle regulation, and proliferation [67], again indicating that CBFB/MYH11 has RUNX1/CBFB repression-independent activities that may contribute to leukemogenesis.

CBFB/MYH11 rearrangements co-occur with RAS gene, or RAS-regulating gene mutations that lead to RAS pathway activation, in more than 90% of CBFB/MYH11 patients, indicating that changes to this pathway play an important role in AML pathogenesis [68]. In addition to RAS genes, whole-genome and whole-exome sequencing analysis of adult and pediatric leukemia samples identified recurrent stabilizing mutations in *CCND2* [69], the gene encoding cyclin D2. All the mutations surrounded the codon for a conserved phosphorylation site (Thr280) that regulates ubiquitination of Lys270 and degradation by the proteasome [70]. The mutations led to increased stability of the CCND2 protein [69] and suggest that stabilization of CCND2 is a previously unidentified and potentially targetable mutation in CBF-AML.

BCR/ABL

The BCR/ABL gene fusion is the result of the t(9;22), and although present in approximately 20% of B-ALL cases is most often associated with chronic myelogenous leukemia (CML). The translocation generates the BCR-ABL fusion protein, in which the N-terminal domains of BCR are combined with almost the entirety of ABL, a nonreceptor tyrosine kinase, whose activity is tightly regulated in normal cells. The oligomerization domain contributed by BCR constitutively activates ABL tyrosine kinase activity and is essential for BCR-ABL transformation.

Depending on the site of the breakpoint in the *BCR* gene, the fusion protein can vary in size from 190 to 230 kDa. Each fusion protein contains the same portion of the ABL protein but differs in the length of the BCR portion. In two-thirds of ALL cases with BCR/ABL, the breakpoint in *BCR* occurs in the region known as the minor-breakpoint cluster region (m-bcr), resulting in a fusion protein of 190 kDa (p190). In CML, however, the *BCR* breakpoint occurs further upstream, in a region referred to as the major breakpoint cluster region (M-bcr), resulting in a protein of 210 kDa (p210). A constitutively active ABL is produced by both the p190 and p210 fusion. Due to the well-characterized mechanisms of BCR/ABL oncogenicity in CML, this fusion will not be discussed further in this chapter and readers are instead directed to other chapters within this textbook which deal specifically with CML.

ETV6/RUNX1

The t(12;21) translocation is the most common genetic lesion in pediatric ALL [71], occurring in 17% of patients. The translocation generates a fusion between *ETV6* and *RUNX1* and occurs prior to the onset of immunoglobulin gene rearrangement, giving rise to leukemic blasts that appear to be blocked at the pre-B stage [72]. As noted in the introduction to this chapter, evidence from twin studies has demonstrated that the *ETV6/RUNX1* fusion is most likely an initiating event in ALL, but expression of the fusion alone is insufficient to cause leukemia. Most cases of ETV6/RUNX1 also have deletions in the wild-type *ETV6* allele [73], and mutations in *PAX5* [74, 75].

The fusion gene encodes a chimeric protein that contains the helix–loop–helix domain of the ETV6 transcription factor fused to nearly all RUNX1. The molecular mechanisms underlying ETV6/RUNX1-induced leukemogenesis remain poorly understood; however, there are some commonalities with the other fusions previously discussed, with ETV6/RUNX1 seemingly to inhibit wild-type RUNX1 transcriptional activity through the aberrant recruitment of HDACs and other transcriptional co-repressors [76, 77].

E2A Fusions

B-cell commitment and development require the sequential action of transcription factors positioned within a hierarchical network and which activate or repress genes to specify the B-cell-specific transcriptome. The *TCF3* gene encodes two basic helix–loop–helix (bHLH) transcription factors, E12 and E47, through alternative splicing and both of these proteins play an indispensable role in the earliest defined stages of B lineage development [78, 79]. Known collectively as the E2A proteins, E12 and E47 form heterodimers with other bHLH proteins and bind to the regulatory regions of many genes expressed in early- and late-stage B cells [80–82]. In common lymphoid progenitors, E2A lies in the middle of the transcription factor hierarchy. E2A expression is induced by the macrophage and B-cell-specific transcription factor and E2A itself then stimulates expression of *EBF1* [83]. EBF1, in turn, remodels the promoter region and thereby activates the expression of *PAX5* [84], which definitively

commits lymphoid progenitors to the B-cell pathway. Data from E2A-deficient cells substantiate the pivotal role of E2A in B-cell commitment; E2A$^{-/-}$ cells possess stem-cell-like properties, including promiscuous expression of genes that are normally associated with non-B-cell lineages.

Approximately 5% of all B-lineage ALLs and 25% of cases with a pre-B phenotype possess the E2A-PBX1 chimera. This translocation fuses the transactivation domain of E2A to the majority of the homeobox protein, PBX1, including its DNA-binding domain. While it is speculated that the E2A/PBX1 chimera blocks differentiation of B cells, no leukemic property of E2A/PBX1 has been identified in pre-B cells. It is possible, considering the central role of E2A in the B-cell transcription cascade, that the E2A/PBX1 oncoproteins may act via a dominant-negative mechanism in which they sequester coactivators, thereby disrupting the function of endogenous E2A proteins. Additionally, the fact that the E2A/PBX1 fusions found in human pre-B acute lymphoblastic leukemia all contain the PBX1 DNA-binding domain suggests that direct gene targeting by E2A-PBX1 is critical for the development of ALL.

Nucleophosmin

Mutations in *NPM1* represent the most common genetic change in adult patients with cytogenetically normal AML, and are seen in approximately 35% of cases [85, 86]. The latest WHO classification of AML recognizes AML with *NPM1* mutations as its own separate category [biological and clinical consequences of NPM1 mutations in AML (2017), 2]. *NPM1* mutation, when present alone, is associated with a more favorable outcome, and when co-expressed with *FLT3-ITD*, it is associated with an improved clinical outcome over *FLT3-ITD* mutations alone [87]. Interestingly, *FLT3-ITD* is about twice as frequent in *NPM1*-mutated AML compared to *NPM1* wild-type AML, which may suggest a mechanistic link between these two mutations [85, 86, 88–92]. Recently, whole-genome or whole-exome sequencing of the genomes of 200 adult cases of de novo AML revealed that the most prominent co-occurring mutations in AML are *NPM1, FLT3*, and *DNMT3A* and the combination of the three mutations is associated with extensive loss of DNA methylation, occurring mostly in coding regions. This observation, along with the fact that this co-occurrence occurs at higher frequency than expected by chance, suggests that harboring mutations in all three genes represents a novel subtype of AML [9]. In AML where all three mutations are present, evidence has shown that the *DNMT3A* mutation occurs first, then *NPM1*, and lastly *FLT3* [93, 94]. *NPM1* mutations are usually stable throughout disease and disappear with remission; therefore these could potentially be used to monitor residual disease, or to detect relapse [88, 91, 95].

The protein product of *NPM1*, nucleophosmin (NPM), is normally an abundant, ubiquitous, and highly conserved phosphoprotein that resides primarily in the nucleolus, although it shuttles rapidly between the nucleus and cytoplasm. By shuttling between these subcellular compartments, NPM plays a role in diverse processes including the regulation of centrosome duplication, transport of preribosomal particles and ribosome biogenesis, maintenance of genomic stability, participation in DNA repair processes, and regulation of DNA transcription (reviewed in [92]). In addition, within the nucleolus, NPM has been demonstrated to bind the important tumor suppressor, p53, and to its regulatory proteins, including ARF [96, 97]. The numerous functional domains of NPM (Fig. 12.6) account for its diverse biochemical functions in both cellular proliferation and growth suppression.

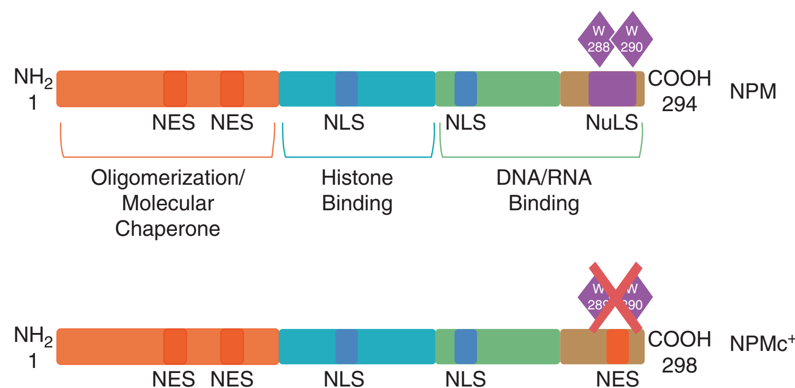

Fig. 12.6 *The functional domains and motifs of wild-type and mutant nucleophosmin*. Wild-type nucleophosmin (NPM, top panel) consists of 294 amino acids that can be subdivided into various functional domains. The cellular localization of NPM1 is dictated by three functional motifs: (1) two leucine-rich nuclear export signals (NES) located in the N-terminal domain (oligomerization domain), (2) a classic bipartite nuclear localization signal (NLS, residues 152–157 and 190–197), and (3) a C-terminal nucleolar localization signal (NuLS) containing two tryptophan residues (W288, W290) which are critical for its nucleolar localization. The mutated NPM1 protein (NPMc$^+$, bottom panel) in AML acquires a new NES at the C-terminus and loses at least one of the two tryptophans in the NuLS. Both alterations are responsible for the exclusion of NPMc$^+$ from the nucleus and its aberrant accumulation in the cytoplasm

In AML, the most common mutations of *NPM1* involve the insertion of four base pairs at the C-terminal portion of the protein. These mutations are unique to AML, and are not seen in other cancers where NPM is usually upregulated through genetic translocations [98]. The C-terminal mutations are usually heterozygous and result in an inability of the protein to perform its usual nuclear-cytoplasmic shuttling function. The cytoplasmic mutant (NPM^{c+}) has been isolated from leukemic blasts [85].

It has been shown that NPM^{c+} relocalizes ARF to the cytoplasm, thus inhibiting its functional interaction with the p53-negative regulator, MDM2, and blunting ARF-induced activation of the p53 transcriptional program. Despite this, the inactivation of ARF by NPM^{c+} is insufficient to cause oncogenesis [97]. This may be because of its dual role as an inactivator of ARF, and an inducer of cellular senescence, as discovered in experiments where NPM^{c+} is overexpressed in fibroblasts [99]. A transgenic mouse model of AML with NPM^{c+} has been generated by placing NPM^{c+} under the human myeloid-specific *MRP8* promoter. In this experimental setting, the cytoplasmic mutation of NPM induces myeloproliferation of mature monocytes and granulocytes but does not induce a maturation arrest [100]. Finally, data from in vitro, cell-free experiments using recombinant NPM^{c+}, patient NPM^{c+}, and leukemia cell lines show that NPM^{c+} reduces apoptosis through an inhibition of caspase-6 and -8 signaling, and inhibits myeloid differentiation also through a caspase-mediated process. Identifying this myeloid-specific function for NPM^{c+} may explain why this mutant is uniquely found in AML [101].

Similar to the model of cytoplasmic ARF sequestration, the $NPM1^{c+}$ mutation causes delocalization of the F-box protein, Fbw7γ, to the cytoplasm, which accelerates this protein's degradation. As Fbw7γ regulates the turnover of MYC, expression of NPM^{c+} ultimately leads to MYC stabilization [102]. MYC overexpression favors myeloid leukemogenesis in mouse models [103]; therefore the resulting elevated MYC expression might also contribute to leukemogenesis in the NPM^{c+} AMLs.

Finally, in the context of wild-type *NPM1* in acute leukemia, a novel function for NPM as a negative regulator of retinoic acid (RA)-induced gene regulation and differentiation toward granulocytes was recently uncovered. In stable RA-resistant acute leukemia cells, NPM is highly overexpressed and resistance is driven by an aberrant association with a putative co-repressor complex containing wild-type NPM and topoisomerase II beta (TOP2B) leading to recruitment of the chromatin remodeler BRG1 to RA-target genes [104].

Activated Signaling

Mutations in proteins involved in cell signaling transduction pathways typically result in the aberrant activation of these pathways, and provide a proliferative and/or survival advantage to hematopoietic progenitors.

FLT3

FLT3 (FMS-like tyrosine kinase 3) is a transmembrane receptor tyrosine kinase that belongs to the same family, the type III receptor tyrosine kinases (RTKs), as FMS, KIT, and platelet-derived growth factor receptors (PDGFRs) [105–108]. The structure of type III RTKs consists of five extracellular immunoglobulin-like domains, a transmembrane and juxtamembrane domain, and an intracellular kinase domain [109].

FLT3 has been found to be mutated in 25% of all AML, and to be overexpressed in some cases of B- and T-cell precursor ALL [110]. In AML, the recurrent mutations include in-frame internal tandem duplication (ITD) of the transmembrane domain in 95% of cases, and a tyrosine kinase domain (TKD) mutation at aspartic acid residue 835 in the remainder [111]. Other transmembrane and kinase domain mutations have been reported at a much lower frequency. For patients with AML with normal cytogenetics, the presence of an FLT3-ITD mutation is associated with a poorer response to therapy and poorer overall survival [87]. It is less clear whether TKD mutations are associated with a poor outcome (reviewed in [111]).

In normal hematopoiesis, binding of FLT3 ligand to FLT3 results in dimerization of the receptor and its activation. In combination with other growth factors, activation of FLT3 results in expansion of the hematopoietic stem/progenitor cells in vitro. For all but dendritic cells, once hematopoietic cells differentiate, the expression of FLT3 is lost [112, 113]. The proliferative role of FLT3 is further supported by knockout mouse models of FLT3 or FLT3 ligand. These mice have a normal life expectancy but show subtle deficits in hematopoietic cells, including a reduction in the number of B progenitor cells, NK cells, and dendritic cells, and reconstitute lethally irradiated mice with only 25% of the efficiency of bone marrow from wild-type mice [114, 115]. Thus, although FLT3 contributes to hematopoietic proliferation, its function is not essential.

In AML, there is constitutive activation of FLT3 due to either interference with the negative regulatory function of the juxtamembrane region with ITD mutations or changes in the activation loop with TKD mutations [116]. As a result, there is autophosphorylation and direct or indirect phosphorylation of several proteins that in turn activate the PI-3-kinase/AKT, RAS/MAPK, and STAT5 pathways, ultimately inducing cellular proliferation, and inhibiting apoptosis (reviewed in [117]).

It is postulated that FLT3 mutations allow for unrestricted growth of the leukemia stem cells. In support of this hypothesis, it has been shown that retroviral transfection of primary murine bone marrow cells with human AML *FLT3* mutation does not cause leukemia, but rather results in an oligoclonal myeloproliferative disease manifested by splenomegaly and leukocytosis [118]. Full development of leukemia occurs when *FLT3-ITD* mutations are

combined with other genetic alterations known to inhibit hematopoietic differentiation, including PML/RARA, NUP98/HOX, RUNX1/CBFA2, MLL/ENL, and MLL/SEPT6 (reviewed in [117]).

Finally, isolation of a stem cell-enriched fraction from patients with FLT3-ITD AML and subsequent injection of these primary cells into NOD/SCID mice resulted in leukemogenesis with identical FLT3-ITD mutations [119]. These data imply that FLT3-ITD mutations occur at the level of the leukemia stem cell (LSC). However, other clinical evidence suggests that the *FLT3* mutation may occur in a subclone of the LSC, including the finding that in the context of relapsed AML, there is loss of the ITD mutation 16% of the time and loss of the TKD mutation 50% of the time [120, 121].

KIT

Human KIT is located on chromosome 4 and encodes a 145 kD transmembrane glycoprotein. Like FLT3, KIT is a member of the type III RTK family [122]. The kinase activity of KIT is tightly regulated by its ligand, stem cell factor (SCF) [123–125]. SCF binding promotes KIT dimerization and transphosphorylation of KIT at specific tyrosine residues that can serve as docking sites for src-homology-2 (SH2) domain-containing signaling and adaptor proteins [108]. KIT signal transduction plays an important role in the proliferation, differentiation, migration, and survival of hematopoietic stem cells, as well as mast cells, neural crest-derived melanocytes, and germ cells [126].

Point mutations in KIT have been identified in AML. Some of the mutations localize within exon 8 [127] and result in hyperactivation of the receptor in response to SCF [128]. Other KIT mutations cluster within exon 17, which encodes the KIT activation loop in the kinase domain [129]. These mutations lead to ligand-independent activation of the kinase domain. KIT mutations are also found in other human malignancies, such as gastrointestinal stromal tumors [130], mastocytosis [131], and germ cell tumors [132].

KIT mutations are observed mostly in association with CBF-AML [133, 134]. It may be that the KIT mutations in this subset of AML provide the myeloid blasts with the necessary extra hit by conferring a proliferative and/or survival advantage, because, as discussed previously, the chimeric transcription factor blocks differentiation, but has a limited effect on cellular proliferation [38–41].

The prognostic significance of KIT mutations in CBF-AML is uncertain [135–138]. A recent meta-analysis evaluating the impact of *KIT* mutations on the prognosis of CBF-AML found that *KIT* mutations had no effect on complete remission (CR) rates, but they resulted in a significantly increased relapse risk [139].

Myeloid Transcription Factors

Hematopoietic stem cells (HSCs) are pluripotent stem cells that give rise to all the circulating blood cell types. They are defined by their ability to self-renew while generating differentiated daughter cells [140]. The maturation of HSCs into blood cells has been suggested to follow a hierarchical model, in which the stem cells undergo a reduction in self-renewal potential while simultaneously gaining functional specialization [141]. Several key transcription factors (TFs) maintain HSC self-renewal, whereas others specify differentiated hematopoietic lineages. As discussed earlier, chromosomal translocations that disrupt the normal functioning of these essential TFs are one way that hematopoiesis becomes deregulated. However, beyond fusions involving these transcription factors, mutations altering their functions have also been uncovered with new sequencing technologies [9, 75].

CCAAT-Enhancer-Binding Protein Alpha (CEBPA)

CEBPA is a tumor-suppressor gene that encodes a transcription factor associated with inhibiting cell proliferation. Mutations in this gene have been reported in 7–16% of AML patients, with a close to equal distribution of patients with either a single- or a double-*CEBPA* mutation. AML patients with a *CEBPA* mutation have improved prognosis versus wild-type patients, and those with a double mutation have better overall survival compared to those with a single mutation. Of note, this improvement in survival is lost if there is a co-occurring FLT3-ITD mutation [87, 142–148].

Mutations in *CEBPA* can occur across the whole coding region; however, two clusterings of mutation hot spots have been identified. The more common N-terminal mutations increase the use of an alternative initiation codon that then leads to the formation of a short p30 isoform, which inhibits the function of the full-length protein by a dominant negative mechanism [145]. C-terminal mutations are generally in-frame insertions/deletions in the DNA-binding or basic leucine zipper domains that disrupt binding to DNA or dimerization. The less frequent C-terminal mutations are generally in-frame insertions/deletions that prevent CEBPA DNA binding via alteration of the protein's basic-leucine zipper (bZIP) domain [149].

Recently, whole-exome sequencing detected a high frequency of *CEBPA* mutations in a subtype of AML, acute erythroid leukemia (AEL) [150]. Significantly, the biallelic *CEBPA* mutations co-occur with *GATA2* mutations. GATA2 is a transcription factor that is crucial for hematopoietic development. Considering the prominent expression of GATA2 and CEBPA in early hematopoietic progenitor cells and the direct protein-protein interaction between GATA2

and CEBPA proteins, further studies are needed to clarify whether and how mutations of both genes contribute to the pathogenesis of AEL [151].

Paired Box Protein 5 (PAX5)

B-progenitor acute lymphoblastic leukemia (B-ALL) is a common pediatric malignancy. A genome-wide analysis of leukemic cells from B-ALL patients using high-resolution single-nucleotide polymorphism arrays and DNA sequencing was performed to identify cooperating oncogenic lesions in this disease. *PAX5* was found to be the most frequent target of somatic mutations, with lesions found in ~30% of the cases analyzed [75]. PAX5 is the essential regulator of B-cell identity and function. Consistent with this view, *PAX5*-deficient pro-B cells, unlike their wild-type counterparts, exhibit promiscuous gene expression and a lack of lineage commitment [152]. Additionally, *PAX5*$^{-/-}$ pro-B cells have the capacity to differentiate into a variety of non-B-cell lineages, including functional macrophages, osteoclasts, dendritic cells, granulocytes, and natural killer cells, when stimulated with the appropriate cytokines [152]. Similarly, in mice, the absence of PAX5 results in the arrest of B-cell development at a pro-B-cell-like stage [153].

The *PAX5* gene encodes a 52 kDa transcription factor, expressed within the hematopoietic system exclusively in the B-lymphoid lineage. By recognizing DNA through the highly conserved paired domain characteristic of the PAX family of transcription factors [154], PAX5 functions both as a transcriptional activator and a repressor. PAX5 activates expression of B-lineage-specific genes such as *CD19* [155], *BLK* [156], and *CD72* [157] while concurrently repressing lineage-inappropriate genes such as *NOTCH1* [158], *M-CSFR* [152], and *FLT3* [159].

The overall effect of the *PAX5* alterations seen in B-ALL is to reduce or inhibit the level of normal PAX5 functional activity, either because of monoallelic deletions or the generation of altered forms of the PAX5 protein [75]. In the context of leukemogenesis, it is important to note that during the normal development of B cells, *PAX5* is subjected to allele-specific regulation, being predominantly transcribed from only one allele in early progenitors and then switched to a biallelic transcription mode as B cells begin to differentiate [160]. It is tempting to speculate that this allelic type of regulation has been imposed because the levels of PAX5 need to be finely tuned, as subtle decreases or increases may dramatically modify B-cell development. Therefore, the loss of wild-type *PAX5* allele due to the identified mutations would eliminate the possibility to turn on normal biallelic transcription, which may directly contribute to the arrest in differentiation arrest seen in ALL [75].

Chromatin Modifiers

Epigenetic information is deposited by “writer” proteins, such as histone methyl lysine and arginine transferases; removed by “eraser” proteins, such as histone deacetylases and demethylases; and decoded by “reader” proteins adapted to bind to chromatin marks using specific structures such as chromo, bromo, and PHD domains [161]. Together, these “chromatin modifiers” help to integrate signals that lead to meaningful changes in gene expression. Several alterations in chromatin modifiers, such as the “readers” ASXL1 [162–164]; the “writers” NSD1 [165, 166], MLL [167, 168], and EZH2 [169, 170]; and the “eraser” KDM6A [171], repeatedly occur in acute leukemia.

MLL Fusions and MLL-PTD

The MLL (mixed-lineage leukemia) gene is located on chromosome 11q23, and fusions involving this gene are seen in both de novo and therapy-related acute myeloid and lymphoid leukemia. MLL is a large DNA-binding protein ubiquitously expressed in hematopoietic cells, including stem and progenitor populations. Using chromatin immunoprecipitation (ChIP) analysis, MLL has been found to be associated with a subset of transcriptionally active human promoters [172, 173] and with RNA polymerase II, suggesting that MLL has a specific role in the regulation of transcription. A SET domain is located at the carboxy-terminal of MLL, and this domain mediates methylation of histone H3 lysine 4 (H3K4), a histone modification which is associated with transcription at active gene loci [174]. Importantly, in mammals, MLL positively regulates the expression of the homeobox (*HOX*) genes. *HOX* genes are transcription factors that participate in the development of multiple tissues, including the hematopoietic system.

Some of the roughly 50 characterized MLL fusion partners (FPs) can be grouped into families based on cellular localization and function (Table 12.1) [175, 176]. All identified MLL fusions contain the first 8–13 exons of *MLL* and a variable number of exons from the FP gene. Furthermore, another type of *MLL* rearrangement, *MLL-PTD* (partial tandem duplication), is a result of internal tandem duplication of select exons. MLL mutant proteins are always in-frame chimeras that reside in the nucleus, regardless of whether the fusion partner is normally nuclear or cytoplasmic in origin. Despite the large number and functional diversity of the fusion partners, there are some common principles that can be applied to all MLL fusions [175]. First, all MLL fusions retain its amino-terminal domains required for the association of MLL with chromatin, so the fusion proteins are still able to bind DNA. Second, expression of the amino-terminal region of MLL (the region retained in MLL fusions) alone

Table 12.1 Classification of MLL fusion partners

Group	MLL fusion partner	Location	Function
1	**AF4**, **AF9**, **AF10**, **ENL**, **ell**	Nuclear	Putative DNA-binding proteins
2	CBP, p300	Nuclear	Histone acetyltransferases
3	AF1P, **AF6**, AFX, EEN, EPS15, GAS7, LARG	Cytoplasm	Presence of coiled-coil oligomerization domain
4	SEPT2, SEPT5, SEPT6, SEPT9, SEPT11	Cytoplasm	Septin family, interact with cytoskeletal filaments, have a role in mitosis
5	N/A	N/A	MLL partial tandem duplication of exons 5–11 (MLL/PTD)

There are more than 50 known MLL partners; therefore this table is not an exhaustive compilation. The six most common fusion partners are highlighted in bold. *N/A* not applicable

does not lead to myeloid transformation [177], indicating that the fusion partners make critical contributions to the oncogenicity of MLL fusions [178]. Third, MLL fusion proteins enforce continuous expression of the *HOX* genes, *HOXA9* and *MEIS1*, which are normally downregulated during hematopoietic differentiation. This sustained induction appears to be critical for leukemogenesis, since forced expression of *HOXA9* and *MEIS1*, in the absence of an MLL fusion, immortalizes hematopoietic progenitors in vitro and results in AML development in transplanted mice [179, 180].

MLL fusions are hypothesized to disrupt normal gene expression patterns, especially those of the *HOX* genes, maintained by wild-type MLL. However, discerning one unifying mechanism of leukemogenesis by MLL fusion proteins is difficult due to the heterogeneity of the MLL fusion partners. The putative mechanisms of MLL fusion-induced transformation may be more easily characterized by dividing the fusion partners into two broad classes based on their normal cellular localization (either nuclear or cytoplasmic). The fusion partners that are nuclear have been associated with various aspects of transcriptional regulation. Therefore, the fusions involving these proteins may lead to transcriptional deregulation of the *HOX* genes through alterations in the histone modification pattern and chromatin structure. An early insight into this possibility was provided by the characterization of the nuclear MLL/CREBBP fusion [181]. CREBBP (also known as CBP) is a well-characterized global transcriptional activator with intrinsic histone acetyltransferase (HAT) activity. Structure–function analysis demonstrated that inclusion of the portion of CREBBP that contains its HAT domain in the fusion is required for full in vitro transformation and is sufficient to induce the leukemic phenotype in vivo [182]. These data suggest that the leukemic effect of MLL/CREBBP results from the combination of the chromatin association and modifying activities of CREBBP with the DNA-binding activities of MLL. Additionally, although the SET domain, which mediates MLL's H3K4 methyltransferase activity, is consistently lost in all the MLL fusions, several MLL fusion partners, for example AF4, AF9, AF10, and ENL [183], associate with the DOT1L histone methyltransferase that methylates lysine 79 residues in histone H3 (H3K79). Methylation of H3K79 is also associated with positive transcriptional regulation [184]. As different methylation marks may positively control transcription in unique ways [185], the replacement of H3K4 activity in wild-type MLL with H3K79 activity in the MLL fusion complex could perturb transcriptional control. In the case of the MLL-PTD, where the SET domain is maintained, the characterization of a mouse model of *MLL-PTD* demonstrated that MLL-PTD facilitates histone H3K4 trimethylation as well as H3/H4 acetylation within target *HOX* gene promoters [186]. This provides further evidence for an epigenetic mechanism as the underlying cause of *HOX* overexpression.

Some MLL fusion partners are normally localized to the cytoplasm and do not display inherent transactivation properties. However, they do possess structural domains responsible for protein-protein interactions. Therefore dimerization/oligomerization mediated by these domains may create a transcriptional activator complex capable of stimulating gene expression. Experiments have been performed where fusion of the first eight exons of *MLL* to β-galactosidase (a bacterial enzyme) resulted in the development of AML in mice [187]. β-Galactosidase is known to oligomerize but has not known leukemogenic potential suggesting that the mere oligomerization of MLL is sufficient to achieve transforming potential. Examples of translocations that produce fusions where the fusion partner probably imparts oligomerization include MLL/GAS7, MLL/AF1P [188], and MLL/LARG [189]. However, when tested in animal models these fusions develop leukemia after a longer latency when compared with the nuclear fusion partners, which suggests a further specific biological contribution, beyond oligomerization, of the fusion partners in dynamics and penetrance of tumorigenesis.

EZH2

A broad class of protein complexes, known as the polycomb group (PcG), are responsible for writing the histone methylation marks that suppress gene expression. PcG is correlated with transcriptional silencing and trimethylation of lysine 27 of histone H3 ($H3K27me^3$) [190]. In mammals, there are two distinct PcG complexes, PRC1 and PRC2 (Fig. 12.1). PRC2 is the primary writer of di- and tri-methylation of H3K27 [191]. PRC2 is composed of four core components (Fig. 12.7), enhancer of zeste homologue 2 (EZH2), suppressor of zeste 12 (SUZ12), and two WD40 domain proteins, EED and RBBP4 [192, 193]. The catalytic domain of EZH2 is its SET domain, and this has

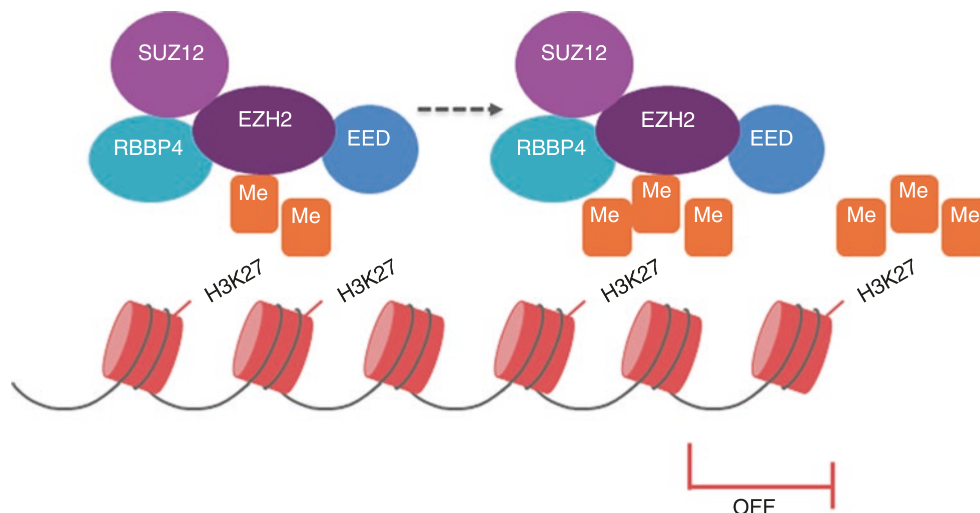

Fig. 12.7 *EZH2 is a histone methyltransferase*. PRC2 is composed of the core subunits, EZH2, SUZ12, RBBP4, and EED. EZH2 catalyzes the addition of methyl groups to histone H3 lysine 27 (H3K27me), ultimately resulting in histone H3 lysine 27 tri-methylation (H3K27me³), a mark associated with transcriptional repression

methyltransferase activity not only toward H3K27, but also weakly toward lysine 26 of histone H1 [194].

EZH2 has a complex role in cancer pathogenesis, acting as an oncogene or as a tumor suppressor, depending on the type of cancer. Evidence in leukemia suggests that it is the loss of EZH2 that contributes to tumor development. In T-cell acute lymphoblastic leukemia (T-ALL), myeloproliferative disorders, and myeloid malignancies, a range of missense, nonsense, and frameshift mutations of *EZH2* occur [169, 170, 195]. These lesions can be heterozygous or homozygous, are found throughout the gene body, and generally are predicted to ablate histone methyltransferase activity via truncation of the SET domain. Loss of EZH2 potentiates oncogenic NOTCH1 and RUNX1 signaling in T-ALL and myelodysplastic syndromes (MDS), respectively [195, 196]. Loss of EZH2 is an indicator of poor prognosis in MDS [169, 170], but the same association with de novo AML cannot be drawn, as EZH2 mutations remain comparatively rare in this setting [197]. While MDS may often progress to AML, this is not to be the case for MDS with EZH2 loss, and genetic deletion of EZH2 in a syngeneic mouse model was shown to prevent the transformation of MDS to AML [196]. Additionally, loss of EZH2 protein occurs in about 45% of relapsed AML samples due to posttranslational deregulation of EZH2 protein [198], and this loss is associated with chemoresistance toward multiple drugs. These findings indicate that inhibition of EZH2 degradation in combination with chemotherapy, and/or targeted inhibitors, may be a novel therapeutic approach in drug-resistant AML.

Cohesin

The cohesin complex is composed of four core components that hold chromatin strands in a ringlike structure that regulates sister chromatid alignment during mitosis (16). The complex has also been shown to play a role in double-stranded DNA damage repair and regulation of transcription (17). Cohesin complex mutations in all four components, STAG1/2, RAD21, SMC1A, and SMC3, have been found in AML and other myeloid malignancies [199–202]. Mutations of cohesin complex components occur in approximately 13% of AML patients [9]. The mutations are almost always mutually exclusive, and occur early in leukemogenesis, at the stage of pre-leukemic hematopoietic stem cells [199].

In most AML cases, cohesion mutations are not associated with genomic instability [9, 203], suggesting that defects in chromatid cohesion do not contribute to leukemogenesis. What, then, are the mechanistic implications of cohesion mutations in leukemia? One hypothesis put forward is that reduced cohesion function alters chromatin structure, disrupting *cis*-regulatory architecture of hematopoietic progenitors and impairing hematopoietic differentiation [204–206]. In agreement with this, using an assay for transposable-accessible chromatin combined with high-throughput sequencing (ATAC-Seq), it was shown that there was a global loss of open chromatin in cohesin-mutant-expressing cells, with a concomitant increase in accessibility at specific motifs for key hematopoietic transcription factors [205], including ERG [207], GATA2 [208], and RUNX1 [209], and enforcing stem cell transcriptional programs.

Spliceosome

The spliceosome is a large and complex cellular machine comprised of multiple protein subunits and small nuclear RNAs (snRNAs) used to remove introns from pre-mRNA during constitutive and alternative splicing [210–212]. Mutations in the spliceosome genes *SF3B1*, *SRSF2*, and *U2AF1* have been found in myelodysplastic syndromes (MDS) and myeloid malignancies, including 10–25% of

AML patients, and are enriched in patients who developed AML secondary to MDS [213–215]. The mutated spliceosome genes encode factors that are involved in the recognition of the 3′-end of the intron, and are mutually exclusive of one another in patient samples [214–217], implying that they may contribute similarly to pathogenesis or, alternatively, may not be tolerated by a cell when they co-occur.

Spliceosome gene mutations may be driving leukemogenesis by causing the mis-splicing of pre-RNA from central hematopoietic drivers. In support of this hypothesis, expression of all three mutations in human cell line models, murine cells, and patient samples results in disruption of normal splicing patterns [218–220]. Furthermore, modulation of splicing catalysis via a spliceosome inhibitor reduced leukemic burden in mouse models, indicating that targeting the spliceosome may represent a novel therapeutic strategy in genetically defined subsets of leukemia [221].

Conclusions and Perspectives

Acute leukemia represents a heterogeneous collection of diseases harboring numerous molecular abnormalities. Earlier research identified recurrent fusion proteins directly linked to the pathogenesis of acute leukemia, while more recent work has focused on the role of an ever-increasing number of specific genetic and epigenetic alterations. However, as whole-genome sequencing becomes increasingly accessible for scientists, clinicians, and patients, there is a need for clarification of the prognostic and therapeutic implications of these alterations.

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13 Epidemiology and Hereditary Aspects of Acute Leukemia

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Introduction

Recent projections for the USA estimate that 6590 patients are diagnosed annually with acute lymphocytic leukemia (ALL) and 19,950 with acute myeloid leukemia (AML), while approximately 1430 patients die from ALL and 10,430 from AML [1]. Together these forms of acute leukemia represent about 1.6% of all newly diagnosed cancers and 2.0% of all cancer deaths in the USA [1]. Advances in the understanding of immunology and molecular/genetic features of the acute leukemias along with laboratory improvements in immunophenotyping and cytogenetic characterization have led to the recognition of molecularly defined subtypes of ALL and AML, targeted therapeutics, and recognition of distinct prognostic groups. The most recent World Health Organization (WHO) classification of hematopoietic malignancies considers three major categories of acute leukemia: AML and related myeloid precursor neoplasms, precursor lymphoid neoplasms (encompassing the entities previously known as ALL), and acute leukemias of ambiguous lineage [2]. Consistent with classifications used in cancer registries, to date most epidemiologic investigations have considered all acute leukemias combined or the broad categories of ALL and AML, although an increasing number of studies, especially those of genetic risk factors, examine cases by molecular subtype. Traditionally pediatric acute leukemias, defined either as those diagnosed at 0–14 or 0–19 years of age, have been studied separately from that in adults.

ALL and AML demonstrate substantial differences in incidence patterns by age and risk factors, although some risk factors overlap (e.g., ionizing radiation). Childhood forms of ALL and AML are distinct from those occurring in adulthood with respect to certain molecular (e.g., cytogenetic) features, demographic characteristics (e.g., incidence according to racial/ethnic group), risk factors, leukemogenic susceptibility associated with certain exposures, and prognosis. There also appear to be important differences in the critical time windows for specific leukemogenic exposures [3] and in the relevant contributions of genetic and environmental factors to the etiology of childhood- versus adult-onset acute leukemias. This chapter reviews acute leukemias in terms of their descriptive epidemiology, risk factors for childhood- and adult-onset disease, and hereditary and genetic aspects. Quantitative measures of risk, described as estimated relative risks (RRs), are defined as estimates of the ratios of risks in exposed versus unexposed populations. For the purposes of this chapter, high RRs are considered to be those ≥4, moderate RRs are 2 to <4, and modest RRs are <2.

Descriptive Characteristics of Acute Leukemias Occurring at All Ages

Age-adjusted incidence of leukemia varies internationally by a factor of about six- to eightfold in women and men, respectively, with the lowest rates in Middle and Western Africa and the highest rates in Northern American and Australia/New Zealand [4]. The International Agency for Research on Cancer (IACR), the primary reporting source for international cancer trends, does not subclassify leukemias into acute and chronic, and myeloid or lymphoid, preventing international comparisons of leukemia subclassifications. Rates also vary across racial and ethnic populations within countries. In the USA, for example, rates of ALL are highest among Hispanics [5].

In the USA, the incidence of AML is 2.6 times that of ALL, with age-adjusted incidence rates of 3.6 versus 1.4 cases per 100,000 person-years, based on data from the nine long-standing cancer registry areas of the Surveillance, Epidemiology and End Results Program (SEER-9), 1975–2013 [5]. Incidence is higher among males than females—by

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nearly 40% for ALL and 50% for AML—and lower among blacks than whites—by 36% for ALL and 11% for AML. Despite differences in incidence by sex and race, age-specific rate patterns are similar within each acute leukemia subtype among all sex and race groups (Fig. 13.1a). Leukemia subtypes differ notably in incidence by age: the age-specific curve for ALL is bimodal, with peaks at the youngest and oldest ages, whereas rates for AML rise consistently over the entire age range. Prior to age 20 years, ALL incidence is four times that of AML, but this pattern reverses with age, with AML incidence 11 times that of ALL among individuals ≥60 years of age. Over the past three decades in the USA, age-adjusted rates of ALL and AML have gradually increased among all sex and race groups (Fig. 13.1b).

Relative survival (RS) rates for individuals diagnosed in SEER-9 during 1975–2012 and followed through 2013 are considerably more favorable for patients with ALL than AML, with approximately 81% and 62% 1- and 5-year overall RS, respectively, for ALL, and 39% and 20%, respectively, for AML. Overall RS for ALL and AML is somewhat more favorable for females than males; overall ALL survival is slightly more favorable for whites than blacks, whereas the reverse is true for AML. Over the past three decades, overall ALL and AML survival has improved, with the greatest strides apparent among the youngest age groups. During 1975–1982, 5-year RS for ALL was 61%, 22%, 11%, and 8% for those <20, 20–39, 40–59, and ≥60 years, respectively, and this increased to 88, 47, 32, and 18% in the corresponding age groups during 2005–2012. During these same two time periods, AML 5-year RS increased prominently from 22 to 65% among those <20 years, 13–55% among those 20–39 years, and 10–40% among those 40–59 years, with a less striking improvement of 3–9% among those ≥60 years.

Risk Factors for Acute Leukemia in Children

Chromosomal translocations have been demonstrated to initiate pediatric leukemia in utero based on studies of leukemia arising in identical twins, investigations in newborn blood spots, and the short latency period characterizing pediatric leukemia [6]. Agents causing such translocations have not been identified, and it is suspected that additional molecular

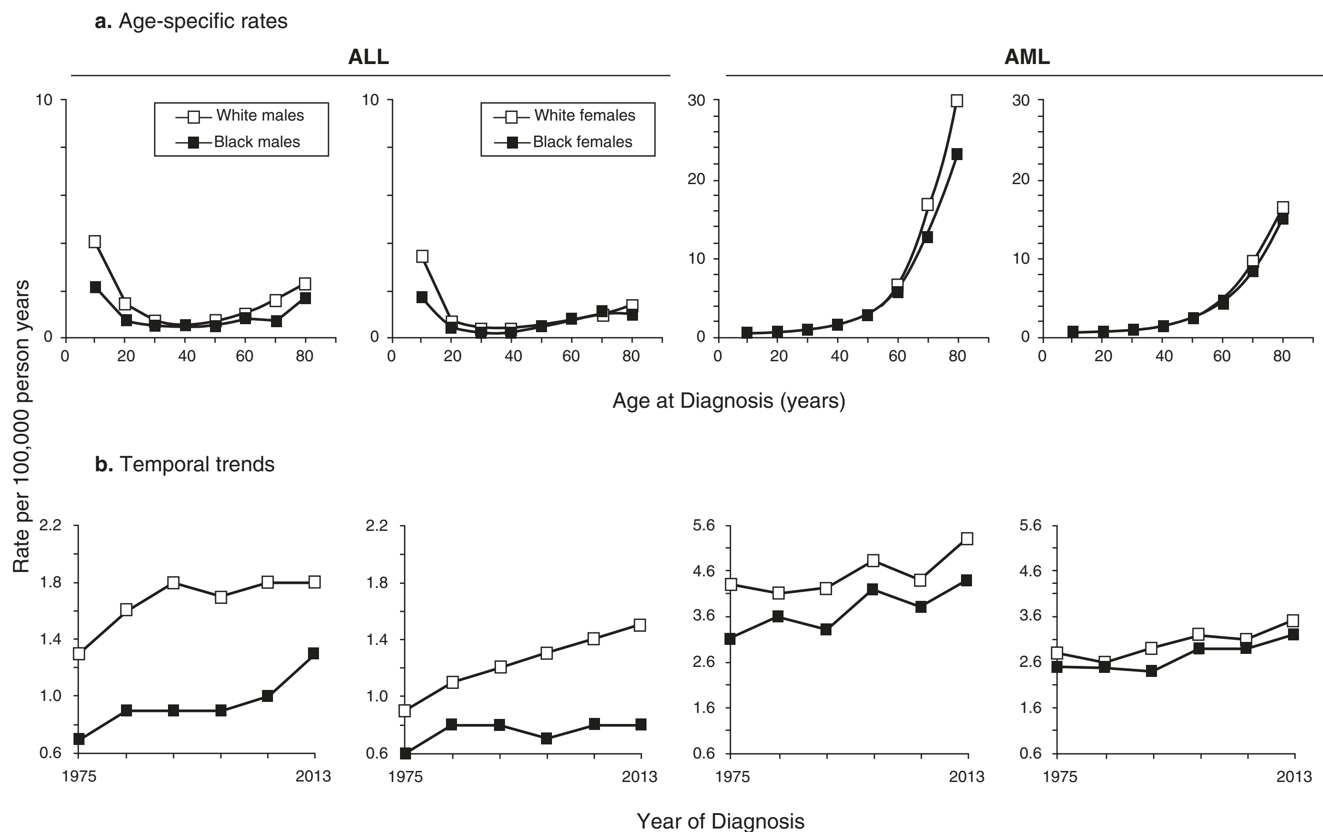

Fig. 13.1 US acute lymphocytic leukemia (ALL) and acute myeloid leukemia (AML) incidence rates (age-adjusted, 2000 US standard) by race and sex, diagnosed among residents of nine cancer registry areas of the Surveillance, Epidemiology and End Results Program, 1975–2013. (**a**) Age-specific rates (<15, 15–24, 25–34, 35–44, 45–54, 55–64, 65–74, 75+); (**b**) temporal trends (1975–1981, 1982–1988, 1989–1995, 1996–2001, 2002–2007, 2008–2013)

changes are required for pediatric leukemia to develop. Data supporting the requirement for additional events in pediatric leukemogenesis include twin concordance rates and results from animal studies. Notably, there is ongoing debate on the frequency at which these translocations occur in healthy newborns [7–9]. Next, we summarize the epidemiologic findings on preconception and prenatal risk factors followed by results for postnatal leukemogenic factors.

Prenatal and Preconception Factors

Reproductive History

Long-standing efforts to identify reproductive factors associated with risk of pediatric ALL and AML have not yielded clearly established etiologic factors. Associations of prior maternal fetal loss with increased risk of acute pediatric leukemias [10–12] were not confirmed in subsequent studies of pediatric [13, 14] or infant [15] leukemia. Although results from individual studies have been inconsistent, a recent meta-analysis reported increased risk of both ALL and AML for older maternal age and increased risk of AML for offspring of young mothers [16]. In the same analysis, older paternal age was associated with ALL while younger paternal age was associated with AML. Inconsistent findings also have been reported for birth order, although two recent large, independent pooled analyses showed slight reductions in risk for ALL with increasing birth order [17, 18].

Medical Conditions and Treatments

Decades of efforts to identify infectious agents causing pediatric ALL have been unsuccessful, and more recent studies of maternal medical conditions and/or treatments generally have not been replicated. It has long been postulated that a viral agent infecting a susceptible mother during pregnancy may play an etiologic role in pediatric leukemias [19]. However, reports linking in utero influenza infection [20] and Epstein–Barr virus [21] with pediatric leukemia and ALL, respectively, were not subsequently confirmed [22–24], and specific leukemogenic infectious agents have not been identified [24–27].

Inconsistent findings have been reported for both maternal hypertension [28–31] and diabetes [11, 28–30]. Observations requiring replication include findings of excess risks of pediatric common B-cell precursor leukemia in offspring of women with a previous molar pregnancy [14] and those receiving antibiotics [32] or mind-altering drugs during pregnancy [33], pediatric AML in offspring of mothers with polyhydramnios or anemia [14], and pediatric hematopoietic malignancies in offspring of mothers who had undergone fertility treatment [13, 34, 35]. An excess of pediatric leukemia in mothers using marijuana before or during pregnancy [36] was not confirmed [37].

Medical Radiation Exposures

Diagnostic X-ray exposures from the late 1940s through the mid-1970s have been associated consistently with modest elevation in the risk of pediatric leukemia, although risks have declined over time as radiation doses have decreased [38, 39]. Increased risk of pediatric leukemia was first linked with fetal exposure to abdominal or pelvic diagnostic X-ray of the pregnant mother nearly 60 years ago [40]. Subsequently, additional data from the large Oxford Survey of Childhood Cancers, a medical record-based study in hospitals in the northeast UK, and close to 30 other case–control studies support an overall RR of 1.4 (i.e., 40% increased risk) for pediatric leukemia associated with diagnostic ionizing radiation exposure in utero, but the interpretation of the statistical association has been debated [39]. Data are insufficient to determine risks of pediatric leukemia in offspring of women undergoing radiotherapy for cancer, and there is no evidence linking ultrasonography during pregnancy with risk of pediatric leukemia [38].

Environmental Ionizing Radiation Exposures

Data evaluating leukemia risk with in utero exposure to environmental sources of ionizing radiation are limited. The cohort study of survivors who were in utero at the time of the atomic bombings of Hiroshima and Nagasaki "… cannot provide information on the effect of radiation on the incidence of childhood cancers" in the absence of complete information for the first 5 years of follow-up and insufficient size to assess such rare outcomes [41]. Similar limitations characterize a cohort of persons who were in utero at the time of the Chernobyl accident [42]. Existing data are not informative about pediatric leukemia risks in offspring of female airline crew or women living in proximity to nuclear testing or nuclear installations [43]. However recent population-based studies have reported elevated risk of childhood leukemia associated with residential gamma radiation [44, 45], but not radon [44, 46].

Parental Occupational Radiation Exposures

A small study linking paternal preconception occupational exposure of nuclear industry workers with excess leukemia risks in offspring [47] was not confirmed in a study of 39,557 children of male nuclear workers [48]. Furthermore, there

was no excess of pediatric leukemia in offspring of female or male medical radiation workers in the UK [49] or the USA [50]. A meta-analysis of all studies of parental occupational exposure to extremely low-frequency magnetic fields (ELF-MF) revealed no association with the risk of childhood leukemia for either maternal or paternal exposure [51].

Parental Pesticide Exposures

Risks of leukemia in offspring of parents occupationally exposed to pesticides have been extensively investigated, but few investigations have identified the specific pesticides to which the fetus has been exposed. Meta-analysis of more than 30 case–control and 5 cohort studies revealed similarly increased risks for both ALL and AML (RR = 2.64, 95% CI = 1.00–5.00; RR = 2.64, 95% CI = 1.48–4.71, respectively) with maternal occupational exposure, with higher risks in studies of farm-related exposures compared with studies of mixed or unknown place of exposure [52–55]. No consistent association was observed for childhood leukemia with paternal occupational exposure to pesticides [54]. A subsequent pooled analysis of 13 case–control studies reported elevated risk of ALL following paternal occupation exposure (OR = 1.20, 95% CI = 1.06, 1.38) and increased risk of AML following maternal occupational exposure (OR = 1.94, 95% CI = 1.19, 3.18) [56]. A recent pooled analysis of home pesticide exposure found 40–55% increased risk for ALL and AML for the preconception and pregnancy time periods [57].

Parental Chemical Exposures

Findings from epidemiological studies of pediatric leukemia in offspring of parents exposed to heavy metals (including arsenic), polychlorinated biphenyls, dioxin, indices for outdoor air pollution, traffic density, drinking water disinfection by-products, and specified and unspecified solvents are generally limited by a scarcity of data available. However some pooled studies and meta-analyses have recently become available for these exposures. A meta-analysis of benzene exposure, and established risk factor for adult leukemia, revealed an association between maternal and paternal exposure and both ALL and AML, with a particularly strong effect for AML (OR = 2.34, 95% CI = 1.72, 3.18) [58]. Pooled analyses from the Childhood Leukemia International Consortium (CLIC) examining occupational [59] and home [60] paint exposure reported null associations for occupation exposures and modest positive associations for home exposure (OR = 1.54, 95% CI = 1.28, 1.85 and OR = 1.14, 95% CI = 1.04, 1.25 for exposure preconception and during pregnancy, respectively). Limited evidence has linked pediatric leukemia in offspring with parental exposure to motor vehicle emissions [61, 62], driving or inhaled particulate hydrocarbons [63], or maternal exposure to unspecified solvents [64]. Maternal exposure to petroleum products during pregnancy has also been linked to infant AML [65].

Parental Cigarette Smoking

The role of maternal smoking in the development of pediatric leukemia has been evaluated for several decades, with a few studies reporting an increased risk. However, a meta-analysis [66] and subsequent studies concluded that maternal smoking during pregnancy is not a major risk factor for childhood acute leukemia [67–69]. Paternal preconception smoking has been investigated less extensively, but a recent meta-analysis of 20 studies showed a modest association with childhood ALL (RR = 1.25, 95% CI = 1.08–1.46) [70].

Parental Alcohol Consumption

A comprehensive assessment of pediatric leukemia risk and parental alcohol consumption in 33 case–control studies did not strongly support an association [71]. Paternal preconception consumption of alcohol has also not been linked with elevated risk [72]. However, a meta-analysis of 21 case–control studies concluded that maternal consumption of alcohol during pregnancy was associated with increased risk of pediatric AML (RR = 1.56, 95% CI = 1.13–2.15), but not ALL [73]. The association between maternal alcohol consumption and AML has also been reported in a subsequent study [69]. Reasons why in utero exposure to alcohol increases AML but not ALL risk are unknown.

Maternal Diet and Vitamin Supplements

The possible role of maternal diet or vitamin supplements in risk of acute leukemia has been investigated by several studies, although heterogeneity between dietary components studied and exposure categories used makes drawing conclusions difficult. Four investigations found reduced risks of pediatric leukemia in offspring of mothers who consumed higher levels of fruits and vegetables during pregnancy [74–77]. A recent pooled analysis reported reduced risk of ALL associated with prenatal vitamin supplementation and weak evidence for a reduced risk of AML associated with maternal vitamin use [78], and a subsequent study reported similar results for both ALL and AML [79]. Other studies also found reduced risk with maternal use of iron supplements [33, 80]. Spector et al. found that maternal consumption of foods that inhibit DNA topoisomerase II was linked with increased risk

of infant AML [74]. Finally, a meta-analysis linked maternal coffee consumption to increased risk of both ALL and AML and cola consumption to increased risk of ALL, whereas tea consumption was associated with decreased risk of overall leukemia in offspring [81].

Mode of Birth

Mode of birth has recently become an exposure of interest for several long-term outcomes in offspring. Support for a role of mode of birth in immune system development includes observations that cesarean delivery alters both the composition [82] and diversity [83] of intestinal microbiota, and these differences can persist through the first 12 months of life [84]; reported associations between cesarean delivery and immune-related disorders, including type I diabetes mellitus [85], asthma [86], and allergies [87]; and evidence that infants born by cesarean delivery experience a markedly diminished stress response before birth, including reduced levels of cortisol and catecholamines [88–90]. A recent pooled analysis of 13 studies found an association between pre-labor cesarean delivery and risk of ALL (OR = 1.23 95% CI = 1.04, 1.47) but not AML [91].

Postnatal Factors

Birth Weight

High birth weight has been linked consistently with increased risk of ALL, and recent studies suggest a similar relationship for AML. A meta-analysis of 31 studies demonstrated significantly increased risks of ALL (RR = 1.23, 95% CI = 1.15–1.32) associated with birth weight of ≥4 kg, and seven of these studies showed a similar relationship for AML (RR = 1.40, 95% CI = 1.11–1.76) [92]. It has been suggested that accelerated growth rather than high birth weight per se is etiologically linked with ALL, and a recent pooled analysis of 12 case–control studies found evidence of moderate increased risk of ALL for large-for-gestational-age infants and those with a higher proportion of optimal birth weight [93]. Low birth weight (<1.5 kg) has been associated with increased risk of AML (RR = 1.49, 95% CI = 1.03–2.15) but not ALL [92].

Breast-Feeding

Large case–control studies examining the association of breast-feeding and risk of childhood acute leukemias have reported 9–20% decreased risk for ALL, but a less consistent picture for AML, ranging from no reduction to 23% reduction in risk [94, 95]. A meta-analysis found that any breast-feeding for 6 months or more was associated with an 18% reduced risk of ALL based on 11 studies and no reduction in risk of AML based on 6 studies [96]. A subsequent pooled analysis of 11 studies found a similar reduction in the risk of ALL for children who were breast-fed for 6 months or more (OR = 0.86, 95% CI = 0.79, 0.94) [17]. Although few studies have examined risks for combined breast-feeding and milk supplementation, one reported a moderately increased risk of childhood leukemia among those infants who received milk supplementation with breast-feeding more than 50% of the time [97].

Medical Conditions and Treatments: Infections, Vaccinations, Allergy, and Atopy

Clusters (a group of cases representing an excess intensity within the population at risk which is unlikely to be due to chance) of pediatric leukemia have been described for decades, but no infectious or other causal agent(s) have been linked with these clusters [98, 99]. A comprehensive, broad-based epidemiologic and laboratory investigation of the largest pediatric acute leukemia cluster to date, diagnosed in residents of Churchill County, Nevada, was unable to identify a causal agent [100].

Two mechanisms have been suggested by which infectious agents might be associated with the age peak from 2 to 5 years in the common form of pediatric ALL. Greaves postulated a two-hit model of leukemogenesis, with the first event or mutation occurring in rapidly dividing immature B cells in utero and the second event arising in early childhood as a result of delayed exposure to infectious agents [101]. Epidemiologic studies of proxy measures of early-life exposure to infections, including daycare attendance [17, 102], birth order [18], and timing of birth [103], generally support a reduced risk of ALL associated with early exposure to infectious agents. However, studies of clinically diagnosed infectious episodes during infancy are not consistent [17, 104].

Kinlen proposed that childhood ALL occurs as a rare response to a specific, albeit currently unknown, viral agent(s), particularly when there is mixing of rural (or other low-density) populations with urban (or other high-density) populations [105]. Summarizing multiple extreme examples of population mixing in Britain, Kinlen observed significant short-term excesses of pediatric leukemia [106], although others reviewing the population mixing studies have disagreed with the conclusions drawn by Kinlen [107].

A limited number of studies investigating an infectious etiology for pediatric acute leukemia have reported a protective effect from vaccination in infancy or early childhood, with risks varying according to the type of vaccine and age at

immunization [108]. A meta-analysis of epidemiologic studies examining atopy and risk of childhood leukemia revealed a 31% significantly reduced risk of ALL based on six studies and no significant association for AML based on two studies [109]. Inverse associations were seen for ALL with asthma, eczema, and hay fever, but the authors caution about overinterpretation of the results in light of the limited number of studies, substantial heterogeneity, and potential misclassification. Finally, a meta-analysis based on eight studies of allergies and ALL reported a 33% reduction in risk [110].

Medical Radiation and Chemotherapy

In general, studies of low-dose postnatal diagnostic radiographic exposures (estimated organ doses range from 0.01 to 6 mGy) have not observed increased risks of pediatric leukemia; the results were based on questionnaire responses and estimated doses were not evaluated [111]. Two reports assessing pediatric cancer risks in children who have undergone computed tomographic (CT) examinations, which are higher dose diagnostic imaging procedures (estimated organ doses from CT scans of the chest, abdomen, brain, spine, and face range from 10 to 80 mGy), reported excess risk of leukemia among children following postnatal CT, with one study reporting a threefold increase in risk (RR = 3.18, 95% CI 1.46, 6.94) for doses of 30 mGy or more [112, 113].

Earlier cohort studies of infants or children irradiated for benign conditions were generally too small to estimate the risk of pediatric leukemia accurately [114], but radiotherapy during childhood for tinea capitis was associated with a subsequent moderately increased risk of leukemia mortality (RR mortality = 2.3, estimated bone marrow dose = 30 mGy) [115]. Two of three earlier small studies found a dose–response relationship between estimated radiation dose to the bone marrow from radiotherapy for treatment of pediatric cancer and subsequent risk of secondary leukemia. A larger more recent investigation did not confirm this relationship; however, excess secondary leukemia was evident—likely due to chemotherapy treatment [116], a strong and well-established risk factor for acute leukemia. More information on the relationship between chemotherapeutic agents and acute leukemia risk is provided in the section of this chapter on adult leukemia.

Environmental Exposures: Ionizing Radiation

Environmental ionizing radiation has been examined as a potential risk factor for childhood leukemia since the establishment of the cohort of Hiroshima and Nagasaki atomic bomb survivors. Investigation of this cohort has demonstrated that survivors who were less than 10 years old at exposure had moderate-to-high relative risks for both ALL and AML [86] [117]. Relative and absolute risks were higher among children than adults, and these risks peaked within 10 years. The absolute risk for incidence was estimated to decrease by about 5% for each year's increase in age, and risk for ALL among females was about 40% of that among males. A recent analysis of this population confirmed excess risk of leukemia among children within the cohort [118].

Results from other studies of postnatal environmental ionizing radiation and risk of pediatric acute leukemias are less clear. Leukemia and lymphoma risks were found to be increased in persons under age 25 among populations living near nuclear fuel reprocessing or weapon production plants in the UK, but not among populations residing close to nuclear plants generating electricity; however, environmental radiation levels measured in proximity to these facilities were considered too low to ascribe to the radiation exposures from these plants [119]. Residential exposure to radon was linked with elevated risk of pediatric ALL in an ecological study but was not associated with an increased risk in studies in the USA [120], Germany [121], France [122], or the UK [123]. Although there was no overall association of residential radon with pediatric AML, a borderline increase was observed in children aged 2 or older [124]. The study from France also revealed no association between childhood leukemia and any type of natural background radiation, including gamma radiation [122].

Environmental Exposures: Nonionizing Radiation

Initial reports of two- to threefold excess risks of pediatric leukemia in children residing in homes with high levels of 60-Hz magnetic field exposures from residentially proximate power lines were assessed further in large studies with more extensive and direct measurements. Results from pooled analyses of these high-quality studies revealed that pediatric leukemia risks were not increased among children residing in homes with power frequency magnetic field exposures under 0.4 μT (which included more than 99% of residences internationally), but were twofold increased among the <1% of children living in homes with exposures ≥0.4 μT [125]. A recent study found that there may be some selection bias in a study of EMF, although no association was found between childhood leukemia and residential exposure to EMF even when accounting for potential biases [126]. Reasons for the suggested increased risk among children with the highest exposure levels are unclear, particularly since experimental studies have not linked power frequency magnetic fields with carcinogenesis [127].

Environmental Exposures: Chemicals

Residential use of pesticides demonstrated a relationship with total childhood leukemia [94] and ALL in two studies, including a large pooled analysis [57, 95, 128], although another study found no association between organochlorine pesticides and leukemia [129]. Exposures to insecticides in agricultural settings were not associated with risk, nor was use of herbicides or fungicides in any setting [64]. There is some evidence that supports a relationship of pediatric leukemia with childhood residential exposure to nearby high traffic density, car repair garages, or gasoline stations [64, 130, 131], and with household use of petroleum products, solvents, and paint [60, 64, 132–134]. No clear association was observed with pediatric leukemia risk and exposure to drinking water disinfection by-products, drinking water nitrate, residence near hazardous waste disposal sites, or exposure to environmental tobacco smoke [64].

Diet and Vitamin Supplements

Only a few small studies have assessed the risk of pediatric leukemia with dietary components, and replication of the results is needed. Findings were mixed for consumption of cured meats [135, 136], and reduced risks were associated with intake of bean, curd, and vegetables in a study in Taiwan [136] and with consumption of foods containing vitamin C and/or potassium in a study in California, particularly during the earliest years of life [135]. Postnatal consumption of cola has not been associated with leukemia [81]. Concern about a report linking intramuscular vitamin K with risk of pediatric leukemia [137] was dispelled following a pooled analysis showing no association [138].

Risk Factors for Acute Leukemia in Adults

Chemotherapy

The development of therapy-related myeloid neoplasms, typically AML (t-AML), is a rare but highly fatal complication of cytotoxic treatments for both malignant and nonmalignant diseases [139–143]. Exposure to such treatments is associated with RRs ≥ 3 and incidence ranging from <1 to 7% for t-AML following conventional therapy, with even higher rates following hematopoietic cell transplantation (HCT) [144].

Therapy-related myeloid neoplasms are classified as a distinct entity in the World Health Organization (WHO) classification system [145], with two types of therapy-related myelodysplastic syndromes (t-MDS) and t-AML differentiated by previous therapeutic exposure. Patients treated with alkylating agents are more prone to t-AML that is preceded by MDS, develops 5–7 years after exposure, and is characterized by loss or deletion of chromosome 5 and/or 7 [−5/del(5q), −7/del(7q)] and presence of somatically acquired loss-of-function mutations in p53 [146]. In contrast, patients treated with topoisomerase II inhibitors, including the epipodophyllotoxins, are more prone to t-AML that is rarely preceded by MDS, arises after a latency of 2–5 years, and is characterized by balanced translocations involving the *MLL* gene at 11q23 [147, 148].

Antimetabolites used for treatment of malignancies (e.g., fludarabine) and for immunosuppressive therapy for autoimmune diseases and transplantation (e.g., azathioprine) have also been shown to increase the risk of t-AML. Abnormalities in chromosomes 5 and/or 7, similar to t-AML associated with alkylating agents, have been described [149, 150]. Population-based studies showing an increased risk of MDS/AML in patients with autoimmune disease have also been published in recent years [151, 152], with evidence suggesting that the association may be attributed to immunosuppressive therapy [153]. Further, solid organ transplant recipients who received azathioprine for initial maintenance immunosuppression had increased risk for both MDS and AML [154].

Although the vast majority of therapy-related leukemias are of myeloid lineage, increased risk of ALL following exposure to chemotherapy has been reported in the literature [155–158]. Several common abnormalities are observed in these cases of t-ALL, including 11q23 abnormalities and t(9;22)(q34;q11) (Ph chromosome) [156–158]. With the introduction and increasing use of a number of new chemotherapeutic agents in recent years, future studies are needed to evaluate the acute leukemia risks and identify cytogenetic abnormalities associated with current treatments.

Medical Radiation

Most epidemiologic studies have not reported a significant association between radiation exposure from diagnostic imaging procedures and AML [159–163]. Use of the radiographic contrast medium, Thorotrast (an alpha-emitting contrast medium with a half-life of 400 years that was used in earlier time periods for cerebral angiography), has been consistently linked with an increased risk of MDS/AML [164–166]. Increased risk of AML has also been associated with radiation treatment for benign conditions, including ankylosing spondylitis [167], benign gynecologic disorders [168, 169], tinea capitis [115], and peptic ulcer disease [170]. Individuals treated with radiotherapy for non-Hodgkin lymphoma, Ewing sarcoma, or cancer of the breast, uterine cervix, or uterine corpus also have moderately increased risks of secondary AML, although the absolute risk is small [114]. Similar to the pattern for primary AML occurring among the

Japanese atomic bomb survivors, initial cases of secondary AML following radiotherapy for another primary cancer often appear within 5 years of treatment (with most cases occurring within 10–15 years) and are associated with estimated bone marrow doses ranging from 1 to 15 Gy [169].

Environmental Exposures: Ionizing Radiation

Compared with Japanese atomic bomb survivors exposed during childhood and adolescence, those exposed in adulthood had lower estimated relative risks of ALL, but the pattern was similar to that following childhood exposure, with risk peaking at less than 10 years since exposure, a declining risk for each year's increase in age at exposure, and risks for women about half the level of risks for men [117, 171]. For AML, relative risks were similar for males and females, but males had two-fold higher absolute risks. A recent analysis suggests that excess risk of acute leukemia mortality continues to persist decades after the bombings [172]. The population living on the banks of the Techa River in the Southern Urals region in Russia, who received chronic low-dose-rate internal and external radiation exposures from releases of radionuclides into the river from the Mayak nuclear weapon plant plutonium production facility, experienced excess risk of leukemias other than chronic lymphocytic leukemia (i.e., non-CLL leukemias) with an excess relative risk estimate of 0.22 per 100 mGy [173, 174].

Occupational Exposures: Radiation

Studies of radiation-exposed workers are important for clarifying the effects of protracted radiation doses, since animal studies suggest that protracted low doses may allow for DNA repair and thus lower risks of leukemia or other cancers compared with risks associated with an acute, single radiation dose [114]. Medical radiation workers were the first population observed to develop radiation-related leukemia within a few years of the discovery of X-rays due to the very high radiation exposures of early workers [175]. This increased risk was largely confined to those working prior to 1960 due to implementation of radiation protection methods in the late 1950s [176, 177]. A meta-analysis of epidemiologic studies in workers who experience low, protracted radiation exposures has shown modest increases in risk for non-CLL leukemias [178]. The International Nuclear WORKers Study (INWORKS), a large international cohort study designed to evaluate cancer risk in radiation-monitored workers, quantified this excess relative risk at 2.96 per Gy for non-CLL leukemia [179]. Leukemia mortality risks were significantly elevated and risk rose significantly with increasing external radiation dose among workers at the Mayak nuclear complex in Russia where effects of plutonium exposures have been under investigation for years [180].

Occupational Exposures: Benzene

For more than 100 years, there has been recognition that occupational benzene exposure is related to risk of leukemia. IARC has determined that benzene exposure is carcinogenic to the bone marrow and causes both AML and MDS, with several potential mechanistic explanations for this association [181]. Currently, the major occupational uses of benzene are in the manufacture of organic chemicals and chemical intermediates [181]. Benzene also occurs naturally in petroleum products and is added to unleaded gasoline [181]. Nonoccupational sources of exposure also exist, with the majority of exposure due to cigarette smoking and emissions from automobiles and industry [182]. A literature review identified 9 cohort and 13 case–control studies that included estimates of benzene exposure, excluded ecologic or proportionate mortality methods, included a comparison group, and assessed risks for one or more subtypes of leukemia [183]. High RRs and a positive dose–response relationship were seen across study designs for AML, especially in more highly exposed workers in the rubber, shoe, and paint industries. Data on ALL were judged to be sparse and inconclusive. A subsequent systematic review and meta-analysis of four studies focusing on cumulative exposure to benzene and AML found a clear dose–response pattern, with RRs of 1.94 (95% CI = 0.95–3.95), 2.32 (95% CI = 0.90–5.94), and 3.20 (95% CI = 1.09–9.45) for low, medium, and high benzene exposure, respectively [184]. A long-standing cohort study of occupational benzene exposure in China has also documented increased risks of MDS/AML in benzene-exposed workers [185–187], with the most recent report showing similar elevated risks in males and females and across different occupations after 28 years of follow-up [188].

Occupational Exposures: Farming and Pesticide Exposure

Increased risks of leukemias and lymphomas have been linked with farming, but relatively few studies have focused on risks of ALL or AML. Some data link occupation as a farmer with increased risk of ALL, but the data are not conclusive [189]. A few studies have examined risks of AML, and results have been mixed. Data from the Iowa Women's Health Study linked residence on a farm or rural area with a small but significant excess of AML, but data were not available to determine if the women worked as farmers or to assess their exposures [190]. In contrast, data from the Women's Health Initiative did not find an association between living on a farm and myeloid leukemia [191].

Exposure to a variety of agricultural chemicals has been evaluated as a risk factor for AML incidence and/or mortality [192–196]. For AML, a significant association was

reported in a meta-analysis of cohort studies evaluating pesticide exposure (OR = 1.55, 95% CI 1.02, 2.34), with evidence that this association was significant for pesticide manufacturers or pesticide applicators but not for agricultural/farm workers [194]. Data from the Agricultural Health Study suggest that the risk of leukemia overall may differ for individual agricultural chemicals, with significant associations detected between leukemia and organochlorine insecticides, fonofos, diazinon, and EPTC [197–200] while no associations being detected for other chemicals [201–203].

Cigarette Smoking

Cigarette smoking was first associated with the risk of adult leukemia in the mid-1980s. Several studies have suggested a small but consistent increased risk of acute leukemia among smokers. In a meta-analysis of studies published through 1992, the estimated relative risks for all leukemia were 1.1 (95% CI 1.0–1.2) and 1.3 (95% CI = 1.2–1.6) for case–control and cohort studies, respectively [204]. A more recent meta-analysis of smoking and AML including studies published through 2013 reported that current smokers (RR = 1.40, 95% CI 1.22–1.60) and ever smokers (1.15, 95% CI 1.15–1.36) have increased risk of developing AML when compared with nonsmokers [205]. Few studies have looked specifically at the risk of adult ALL and smoking; however, studies that have evaluated associations by subtype have typically reported stronger associations for myeloid leukemia [206].

BMI and Diet

The majority of studies have shown a modest, but statistically significant, association between obesity and leukemia, with a recent meta-analysis of 16 prospective studies yielding an adjusted relative risk (RR) for AML of 1.53 (95% confidence interval (CI) 1.26–1.85) and for ALL of 1.62 (95% CI 1.12–2.32) for individuals with a BMI > 30.0 kg/m^2 compared to individuals with a BMI < 24.9 kg/m^2 [207]. A limited number of studies have investigated dietary factors and AML [206, 208–213]. A large prospective cohort study identified higher meat intake with increased risk of AML [212], and this finding was supported by a recent case–control study [211]. Mixed evidence has been reported for associations between AML and fruit and vegetable consumption [206, 208, 211–213]. Alcohol intake has not been shown to be an important risk factor for adult acute leukemia [214–216]. The few studies conducted to date along with methodologic issues related to dietary assessment, particularly in case–control studies, suggest that additional epidemiologic research is needed.

Physical Activity

A recent pooled analysis of 12 prospective cohorts reported a significant reduction in the risk of myeloid leukemia associated with high levels of leisure-time physical activity (HR, 0.80; 95% CI 0.70–0.92) while no significant association was observed for lymphocytic leukemia (HR, 0.98; 95% CI 0.88–1.12) [217]. In contrast, a previous meta-analysis including eight published studies reported no significant association between leukemia overall and physical activity (RR, 0.97; 95% CI 0.84–1.13); however, this analysis did not stratify by subtype of leukemia [218]. Data from the prospective VITamins And Lifestyle (VITAL) study which were not included in either the pooled or meta-analysis also support an association with myeloid neoplasms [219]. Additional studies of AML and ALL will be required to clarify this association.

Nonsteroidal Anti-inflammatory Drug and Acetaminophen Use

Studies of a wide range of malignancies have demonstrated a potential chemopreventive role for frequent nonsteroidal anti-inflammatory drug (NSAID) use. Further, there is evidence that these effects may be specific to certain classes of NSAIDs. Recent evidence from case–control and cohort studies suggests that aspirin use may be associated with reduced risk of AML [220–224]. In contrast, acetaminophen use may be associated with increased risk of AML [221–224]. Given the limited number of studies conducted to date and the small sample size in some studies, further research on the topic is warranted.

Hereditary and Genetic Aspects of Acute Leukemias

Familial Aggregation and Genetic Syndromes

Familial aggregation of acute leukemias is rare. Studies of the few pure familial AML pedigrees have identified germline mutations in *RUNX1* and *CEBPA* [225]. Few studies have described familial aggregation of ALL, although novel familial syndromes involving germline mutation genes frequently somatically mutated in the disease (e.g., PAX5, ETV6) have recently been described [226–228]. Individuals having a twin with ALL have substantially elevated risk for ALL, particularly among monozygotic twins [229], although this risk is due to transplacental migration of preleukemic cells rather than genetic predisposition [230].

A much larger body of evidence regarding the hereditary component of acute leukemia derives from the study of

patients with rare genetic syndromes, but these patients also account for only a small proportion of the total population burden of acute leukemia. Among children with rare inherited bone marrow failure syndromes, including Fanconi anemia, dyskeratosis congenita, congenital neutropenia, and Shwachman-Diamond syndrome, risk of AML is strikingly elevated—often more than 100-fold [231, 232]. The molecular events that predispose to AML among these individuals are not completely understood but are thought to differ by syndrome and involve defective DNA repair, shortened telomere length, and abnormal hematopoietic differentiation and proliferation [233]. Other hereditary conditions associated with increased risk of acute leukemia include Li-Fraumeni syndrome, ataxia–telangiectasia, Bloom syndrome, Noonan syndrome, and neurofibromatosis 1 [234–238].

Myeloid proliferations related to Down syndrome (trisomy 21) have unique morphologic, immunophenotypic, clinical, and molecular features [2]. Children with Down syndrome have a 100-fold risk of AML, as well as approximately 15-fold risk of ALL [239]. Individuals with Down syndrome are disproportionately found to have somatic mutations in the hematopoietic transcription factor *GATA1*, which results in impaired hematopoietic cell differentiation [240].

Genetic Susceptibility

Numerous studies have investigated common genetic variation in germline DNA in relation to leukemia risk. Candidate gene studies of acute leukemias have frequently focused on carcinogen metabolism, folate metabolism, and DNA repair in the past, and have shown some consistency of findings [241]. However, the same genes have not been identified by agnostic methods such as genome-wide association studies (GWAS), which casts doubt on previous findings.

Rather than implicating previously hypothesized pathways or mechanisms, GWAS of childhood ALL have identified risk loci in or near several genes that regulate the transcription and differentiation of B-cell progenitors (*IKZF1*, *ARID5B*, *CEBPE, and GATA3*) and the tumor-suppressor genes *CDKN2A* and *CDK2NB*, which is also frequently somatically altered in hematologic malignancies [242–253]. Although the leukemia risks associated with these loci are low, these inherited genetic susceptibilities can be common in the general population, and it is estimated that common variants identified to date account for about 25% of pediatric ALL [254]. These common variants appear to cause ALL in children with non-European ancestries to a similar degree, but differences in allele frequency may explain a portion of interethnic differences in the disease rate [255, 256]. It is also notable that some variants discovered by GWAS are particularly associated with cytogenetically defined subtypes of ALL, for instance *GATA3* with Ph+-like disease [249]. Additional research is needed to understand the biological basis of these findings, identify other genetic loci that confer acute leukemia risk, and investigate the potential interaction of genetic susceptibility with environmental and individual risk factors.

Summary

Causes of the acute leukemias in children and adults have not been fully identified, but recent progress has provided new etiologic insights. The most well-established risk factors for acute leukemias include ionizing radiation, benzene, and cytotoxic chemotherapy, with increasing risks associated with increasing doses. Among children, risks of pediatric leukemia (ALL and AML) are modestly increased with maternal in utero exposures to diagnostic X-rays in earlier decades, moderately increased with radiotherapy treatments, and moderate-to-highly increased with childhood exposure to the atomic bombings. Among persons exposed as adults, acute leukemia risks are moderately increased among atomic bomb survivors, individuals living near the Mayak nuclear weapon plant, and patients receiving radiotherapy treatments, and AML risks are moderate-to-highly increased with occupational benzene exposure. High risks of AML occur among children and adults receiving cytotoxic treatments (e.g., alkylating agent and epipodophyllotoxin chemotherapy) for benign and malignant disease.

Although pediatric ALL has long been thought to have an infectious etiology, no infectious agent has been isolated. However, recent research has identified modest associations with other environmental, lifestyle, and medical factors for all acute leukemias. Pediatric leukemia has been linked with prenatal maternal occupational pesticide exposure (ALL and AML) and postnatal residential use of insecticides (ALL only). Modest associations have been reported for pediatric AML with maternal prenatal alcohol consumption, pediatric ALL with paternal preconception smoking, and adult AML with cigarette smoking. Anthropometric measures have also been associated with both ALL and AML in children (high birth weight) and adults (high body mass index). Dietary factors may play a role in both pediatric and adult leukemia, but more studies are needed to clarify associations. Interestingly, pediatric ALL risk appears modestly reduced among children who were breast-fed or have a history of atopy.

Recent progress has been made in understanding the germline mutations underlying familial AML, the molecular events by which rare genetic syndromes predispose to the acute leukemias, common genetic variants that confer increased susceptibility to the acute leukemias, and agents causing recurrent chromosomal and genetic abnormalities associated with acute leukemia molecular subtypes.

Although insights into etiology to date have facilitated some prevention efforts for the acute leukemias, additional epidemiologic work is needed to further impact the disease burden worldwide.

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Classification of the Acute Leukemias: Cytochemical and Morphologic Considerations

14

N. Nukhet Tuzuner and John M. Bennett

Definition and Classification

The acute leukemias are a heterogeneous group of neoplasms affecting uncommitted or partially committed hematopoietic stem cells. The origin of the malignant neoplasm is almost invariably within the marrow. Replacement of the marrow pulp or repression of normal hematopoietic cells results in variable degrees of anemia, neutropenia, and thrombocytopenia.

Historically, the term acute implied not only a poorly differentiated blast population, but also a clinical syndrome that led to a rapid fatal outcome. Since it is apparent that, with modern chemotherapy, including bone marrow transplantation (BMT), patients with acute leukemia of several morphologic types can enjoy complete remission (CR), and indeed cure, the term acute can be maintained for nosologic reasons only.

Traditionally, morphology and cytochemistry identified the different involved lineages. During the past 25 years, major advances in our knowledge of the nature of acute leukemia consequent to the application of the techniques of immunology, cytogenetics, and molecular studies have taken place. The demonstration of membrane and cytoplasmic antigen or enzyme by immunologic or immunocytochemical methods and detection of the recurring chromosomal abnormalities either conventional cytogenetic or molecular methods, studies utilizing microarray analysis of gene expression, DNA copy number alterations, and next-generation sequencing provides supplementary arguments for accurate classification.

Identification of subclasses of acute leukemia is important for three reasons. First, some leukemias have clinical features that influence the therapeutic approaches; that is, central nervous system (CNS) is more frequently involved in acute lymphocytic leukemia (ALL) than in acute myelocytic leukemia (AML). Acute promyelocytic leukemia (APL) is associated with intravascular coagulation [1] and acute monocytic leukemia (AMoL) with skin and gum infiltration [2]. Second, differences in response rate and survival are observed in the treatment of acute leukemias; that is, the percentage of CR rate is higher in childhood ALL than in adult ALL. Third, classification greatly facilitates communication and cooperation around the world, and comparisons of results are possible only through reproducible definitions of the acute leukemias.

Examination of both peripheral blood and bone marrow smears is necessary for the diagnosis and the classification of acute leukemias. The most useful cytochemical stains are myeloperoxidase (MPO); Sudan Black B (SBB); specific and nonspecific esterase, such as chloroacetate esterase, naphthol ASD acetate esterase with and without sodium fluoride (NAF), α-naphthyl acetate esterase (ANAE), or butyrate esterase; periodic acid-Schiff (PAS); and acid phosphatase (Figs. 14.1, 14.2, and 14.3).

There are two instances in which the diagnosis of acute leukemia can be made on the basis of histologic material. The first occurs when there is an abundance of reticulin in the bone marrow, resulting in a so-called dry tap [3]. This can be present in any of acute leukemias and is recognized most commonly associated with acute megakaryoblastic leukemia and acute panmyelosis with myelofibrosis. Although touch preparation smears may be of help in such cases, well-prepared hematoxylin and eosin (H&E) stains of paraffin-embedded

One of the coauthors of this chapter (JMB) participated in the WHO Clinical Advisory committee on hematologic neoplasms and coauthored several of the upcoming chapters on MDS and MDS/MPN. The review article by Arber and Hasserjian (Reclassifying Myelodysplastic Syndromes: what's where in the new WHO and why. Hematol. Am. Soc. Hematol. Educ. Program. 2015:294–298) discusses many but not all of the proposed changes. Availability of the chapters was made possible by the WHO to facilitate the writing and revisions necessary.
We anticipate that the entire fascicle will be available in hard copy and via the Internet late in 2017. "We are indebted to the many authors who wrote the revised chapters and have incorporated the changes in this chapter."

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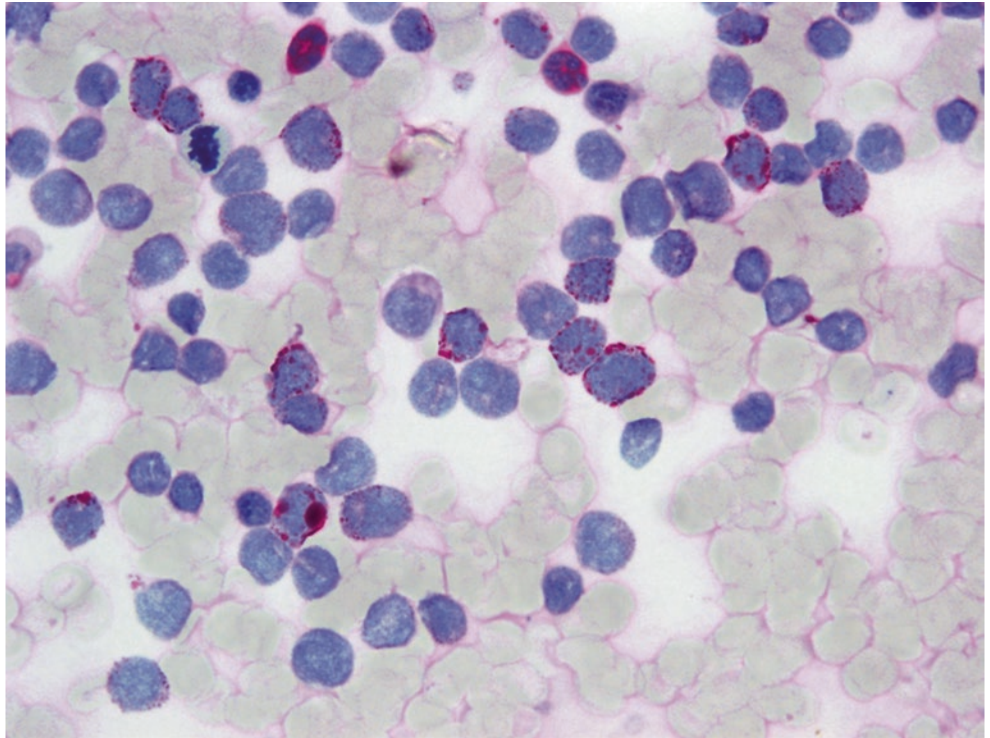

Fig. 14.1 ALL: PAS reaction. Note the blocklike reaction product

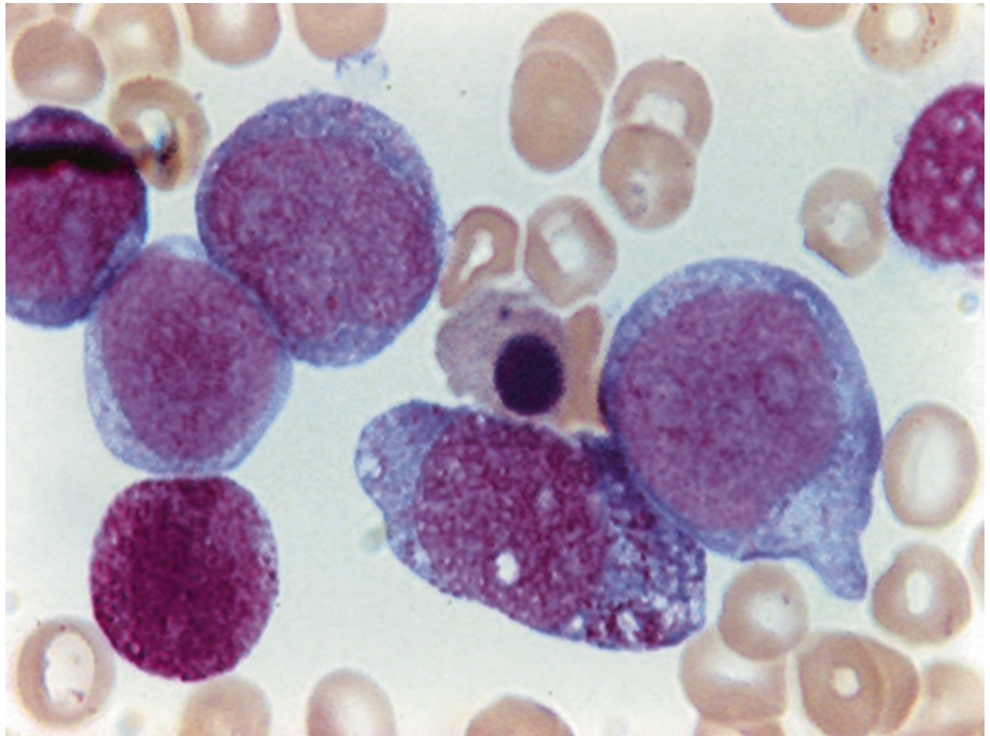

Fig. 14.2 AML (FABM0). Blasts with no differentiation. Peroxidase negative but with positive myeloid antigens by flow cytometry

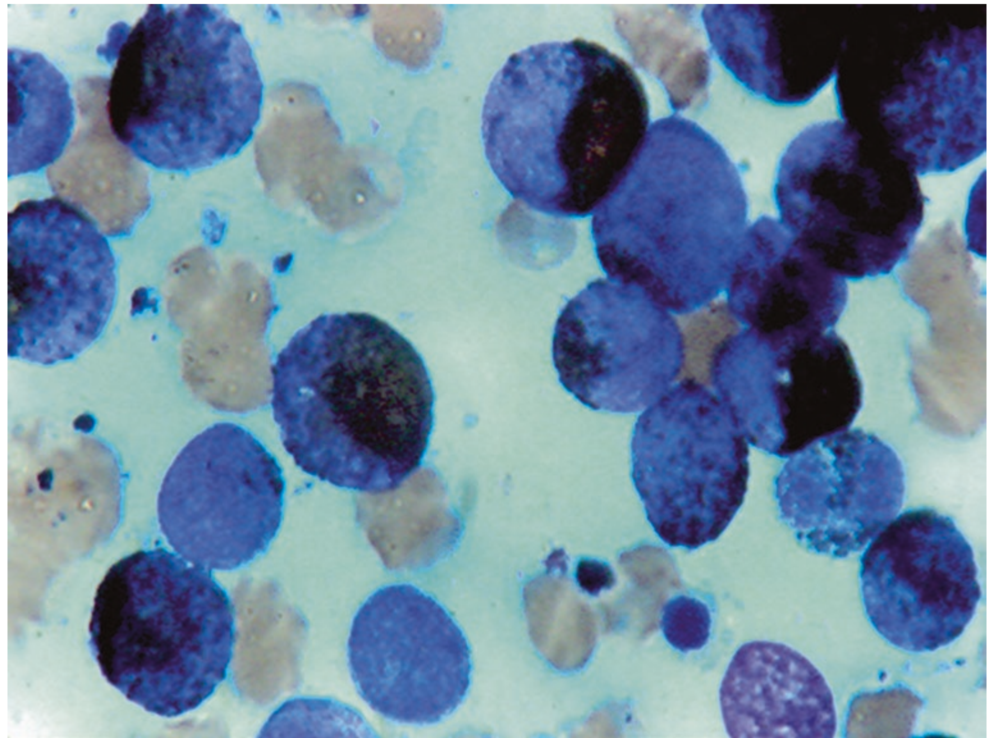

Fig. 14.3 AML (myeloperoxidase stain). *Black* reaction product with Auer rods

material, PAS, chloroacetate esterase stains, and immunologic methods identifying antigens on cell surface (CD41, CD61 or factor VIII) may establish the diagnosis. However, the distinction between acute megakaryoblastic leukemia with myelofibrosis and acute panmyelosis with myelofibrosis may be difficult, particularly if no specimen suitable for cytogenetic analysis can be obtained [4].

The second example is hypocellular (hypoplastic) acute myeloid leukemia in which bone marrow aspirations from several sites and biopsy from one site are necessary for diagnosis [5]. The correct diagnosis depends on a representative biopsy specimen. The third example is that of an uncommon localized extramedullary mass of cells of the granulocytic–monocytic series (myeloid sarcomas) [6]. These tumors can be seen in an established diagnosis of AML, either at presentation or as the first manifestation of relapse.

Diagnosis of Acute Leukemias

The diagnosis of acute leukemia is based exclusively on the morphology of the bone marrow and peripheral blood leukemic cells in the Romanowsky-stained smears. The initial intent was to separate ALL and AML into easily identifiable cell types. In attempting to define boundaries between overlapping groups, major attention is given to the predominant cell type present. An essential cytochemical stain is one that demonstrates MPO, an enzyme restricted to the primary granules of granulocytes and monocytes [7]. Morphologic classification of acute leukemia was that proposed by the French–American–British (FAB) Cooperative group [8], widely used for over two decades. In the meantime, genetic features (cytogenetic, molecular genetic, and gene expression) as well as history of myelodysplasia and prior therapy have been shown to have a significant impact on the clinical behavior of acute leukemias. In 2001 World Health Organization (WHO) proposed a classification in which genetic information was incorporated with morphologic, cytochemical, immunophenotypic, and clinical information into diagnostic algorithms for the acute leukemias [9]. WHO 2008 classification and upcoming revised edition (expected in late 2016) expanded the number of entities with recurrent chromosomal translocations and gene mutations and included new provisional entities [10].

Acute Lymphoblastic Leukemia/Lymphoma (ALL/LBL)

ALL/LBL is one of the most common malignancies observed in the pediatric age group. It derives from the clonal proliferation of lymphoid progenitors in the bone marrow. ALL represents about 80% of cases of acute leukemia in children but only 20% in adults. The consequence of bone marrow infiltration is various cytopenias in the peripheral blood and is associated with the appearance of peripheral blast cells. In some instances, leukemic cells are not seen in the peripheral blood. Thus examination of bone marrow is usually

necessary to confirm the diagnosis. Since the CNS is infiltrated at diagnosis in 5% of patients, examination of the cerebrospinal fluid (CSF) by cytocentrifuge is also necessary.

Morphologic Features

Morphologic classification of ALL was proposed by the FAB group in 1976 [8] and widely used for over two decades. However, the WHO 2008 no longer emphasizes the morphologic classification and separation of the morphologic subtypes. The morphologic features of B- and T-ALL/LBLs are indistinguishable. The lymphoblasts in ALL/LBL vary from small blasts with high nuclear/cytoplasmic (N/C) ratio, scant cytoplasm, condensed nuclear chromatin, a small inconspicuous nucleolus, and a regular nucleus (Fig. 14.4) to larger cell with irregular nuclear membranes, one or more nucleoli, more cytoplasm, and lower N/C ratio (Fig. 14.5). A rare type of leukemia seen in approximately equal percentages in children and adults (about 3–5%) is morphologically identical to the cell characteristics of Burkitt lymphoma. The cells are large and homogeneous with round-to-oval nuclei. The chromatin is finely dispersed with prominent nucleoli. The cytoplasm is intensely basophilic with or without vacuoles (Fig. 14.6). The term B-ALL/LBL should not be used to indicate Burkitt lymphoma/leukemia since it is constantly associated with surface immunoglobulin (sIg) (mature B-ALL) [11]. In addition, an identical nonrandom chromosomal abnormality t(8;14) translocation has been described in both Burkitt lymphoma and leukemia [12]. In rare instances, terminal deoxynucleotidyl transferase-positive (TdT+) B-cell lymphoma/leukemias with BCL2 and/or MYC alterations have been reported [13–15]. These cases do not have the typical morphologic features and chromosomal translocation.

Cytochemistry

By standard definition, the peroxidase reaction is totally negative in leukemic lymphoblasts. SBB stain is also negative. However, it has been suggested that SBB is less specific than MPO because positive reactions have been reported in ALL [16]. It should be remembered that in case of negative MPO or SBB reaction, megakaryocytic, erythroid, some monocytic, and AML with minimal differentiation subtype as well as lymphoid lineage may be involved.

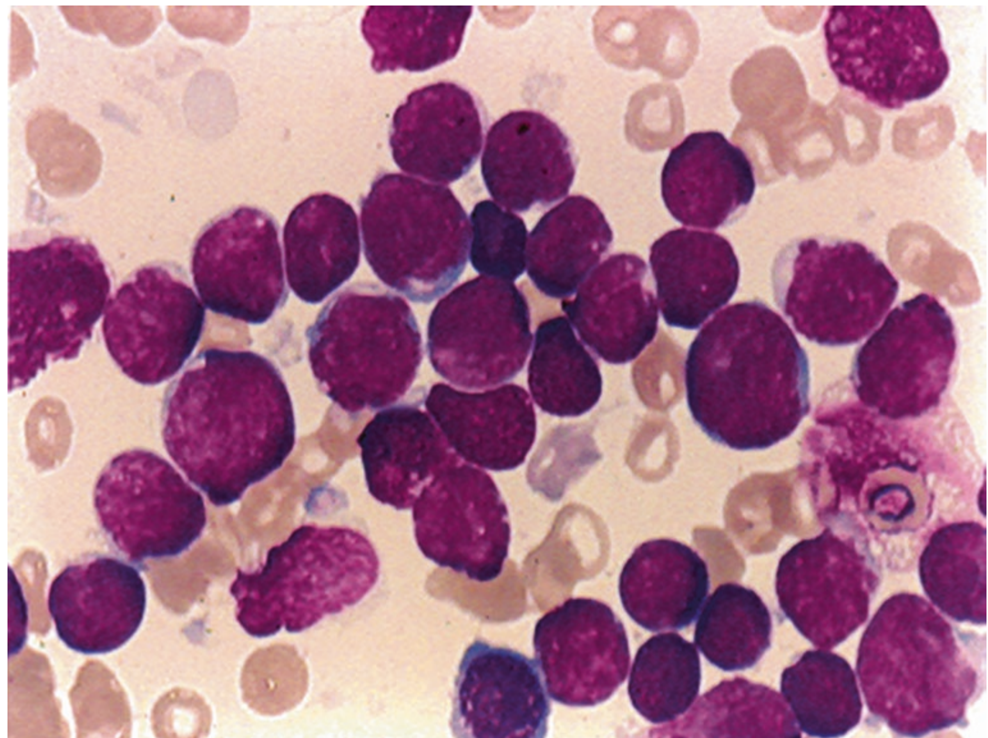

Fig. 14.4 Acute lymphocytic leukemia (ALL): agranular blasts with high nuclear/cytoplasmic ratio; smooth nuclear membrane; rare nucleoli. WG stain

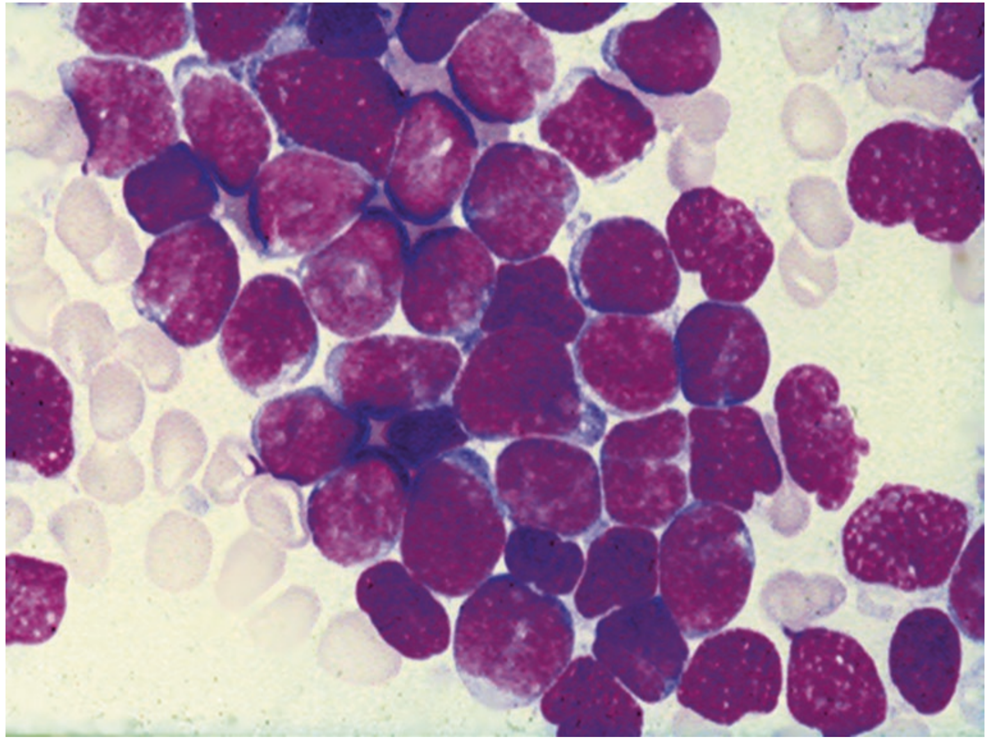

Fig. 14.5 ALL: More cytoplasm than L1 with prominent nucleoli. WG stain

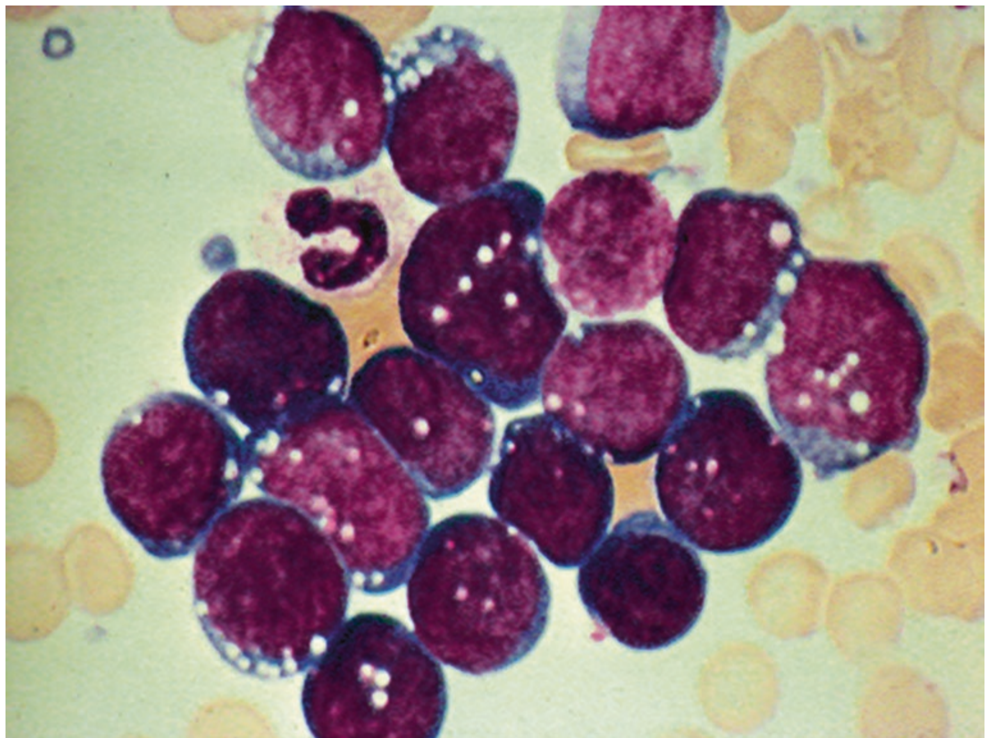

Fig. 14.6 Burkitt lymphoma/leukemia (mature B-ALL): blasts with oval nuclei, finely dispersed chromatin, basophilic cytoplasm with vacuoles. WG stain

The PAS reaction is useful in supporting the diagnosis of ALL (Table 14.1), but its importance has declined, as immunophenotyping has become more important for this purpose. Although blocklike PAS positivity is considered characteristic of ALL, only 15% of cases have this type of PAS reaction (Fig. 14.1) [17]. This pattern of PAS positivity correlates with the immunologic phenotype which is more common in B-lineage cases. The presence of both vacuolated blasts and PAS positivity correlates with reactivity of CD10 antigen [18]. It should be noted that blocklike PAS positivity can be seen in monoblasts in acute monoblastic leukemia and in erythroblasts in acute erythroleukemia [19]. Moreover, fine granular activity in blocks of PAS material can be identified in all AML cell types (Fig. 14.7). PAS stain is therefore a poor discriminator of various cell types, but it may be useful as a part of battery of stains, particularly when many of the reactions are negative or nonspecific. A strong focal (paranuclear) acid phosphatase (ACP) activity or a strong focal ANAE activity greater than 75% of blasts is characteristic of T-ALL [20]. However, it should be remembered that a localized ACP and ANAE reaction is also a feature of rare forms of acute megakaryoblastic and erythroleukemia [21].

Table 14.1 Reaction of acute leukemic cells to cytochemical stains

Stain	AML (M1–M3)	AMML (M4)	AMoL (M5)	ALL (L1–L2)
PAS	±	+	++	++
Acid phosphatase	–	±	++	+++
Peroxidase	+	+	±	–
Chloroacetate esterase	+	+	–	–
α-Naphthyl acetate esterase	±	+	++	++++

PAS Periodic acid-Schiff, − absent, ± occasional activity, + moderate activity, ++ strong activity, +++ focally strong activity in T cells, ++++ focally strong activity at acid pH in T cells

Immunohistochemistry

Indirect immunoperoxidase and indirect alkaline phosphatase anti-alkaline phosphatase (APAAP) techniques are applied to fixed cells in blood and bone marrow smears, cytospin preparations [22], or paraffin-embedded material. These methods permit the detection of surface antigens (i.e., CD19, CD20, CD3, CD1a, and CD10), cytoplasmic antigens (CD79a, MPO, or cytoplasmic μ chain), and nuclear antigens (e.g., TdT and PAX5) [23].

Genetics of Acute Lymphoblastic Leukemia/Lymphoma

Acute lymphoblastic leukemia/lymphoma is relatively homogeneous at morphologic level. However, significant heterogeneity is seen at the genetic level. These genetic lesions define disease subsets with distinct biology and response to treatment and are used in the risk stratification schemas for current protocols [10, 24]. In the last decade,

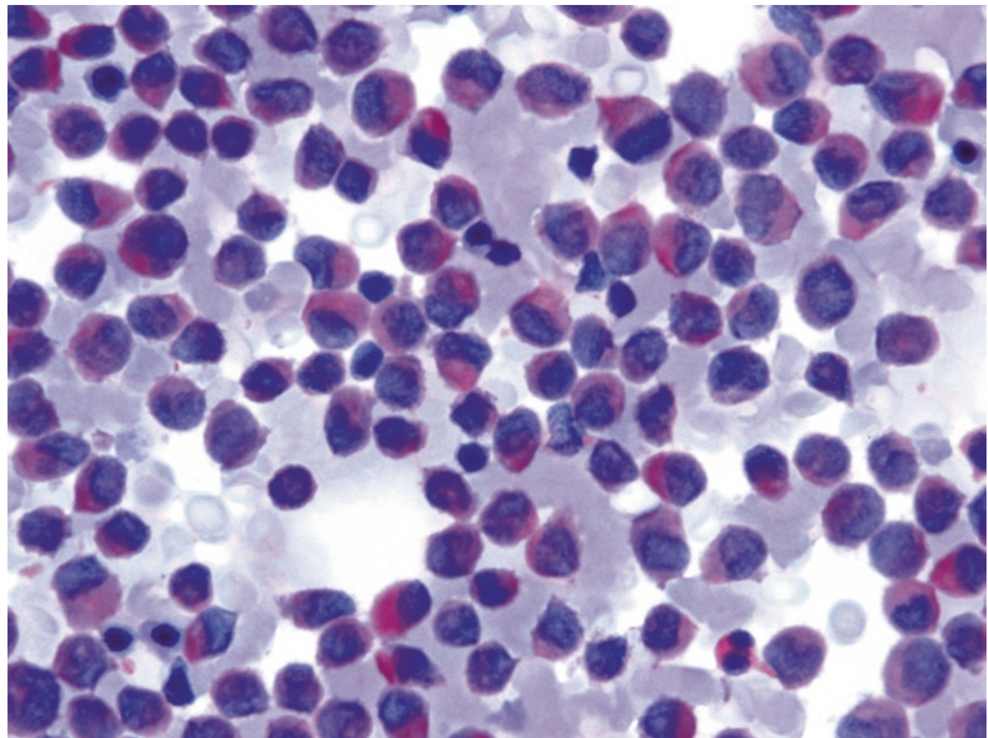

Fig. 14.7 AML: PAS reaction. Note the blush background

studies utilizing microarray analysis and next-generation sequencing have provided major insights into the pathogenesis and clinical behavior of ALL. These studies have identified new ALL subtypes. High hyperdiploidy (>50 chromosomes) occurs in 25–30% of childhood B-ALL and is associated with favorable outcome. Hypodiploidy with 44 chromosomes is uncommon (2–3% of cases) and is associated with poor outcome [25, 26]. Chromosomal rearrangements commonly involve hematopoietic transcription factors, cytokine receptors, epigenetic modifiers, and tyrosine kinases [27]. Common rearrangements in acute B lymphoblastic leukemia are as follows: the t(12;21)(p13;q22) (TEL-AML1) occurs in 15–25% of pediatric cases and is associated with excellent prognosis; t(1;19)(q23;p13) (E2A-PBX1) is seen in 2–6% of pediatric cases, associated with excellent prognosis and CNS relapse; t(9;22)(q34;q11.2) resulting in formation of the "Philadelphia" chromosome (Ph) encoding BCR-ABL1 is associated with 2–4% of pediatric and 25% of adult cases and with poor prognosis; rearrangements of MLL at 11q23 to a range of fusion partners are common in infant ALL (1–2%) with poor prognosis; rearrangement of the cytokine receptor gene CRLF2 is common in Down syndrome-associated and Ph-like ALL and with poor prognosis in non-Down syndrome-associated ALL; t(8;14)(q24;q32), t(2;8)(q12;q24), and t(2;8) (q12;q24) encoding; and MYC rearrangement is associated with Burkitt lymphoma/leukemia which is a neoplasm of mature B cells (2% of cases) and associated with favorable prognosis with short-term high-dose chemotherapy [28, 29]. Approximately 20% of childhood B-ALL cases lack one of these alterations and have alternative sentinel genetic lesions, including deregulation of the ETS family transcription factor ERG, or one of a diverse range of alterations that drive kinase signaling in Ph-like ALL.

T-ALL is also characterized by translocations that deregulate transcription factors, commonly by rearrangement to T-cell antigen receptor loci, and recurring sequence mutations and DNA copy number alterations that disrupt developmental, signaling, and tumor-suppressor pathways, including activating mutations of NOTCH1 and rearrangements of transcription factors TLX1 , TLX3, LYL1, TAL1, and MLL [9, 30, 31].

The finding of prominent eosinophilia either T or B lymphoblastic leukemia/lymphoma must be considered for a rearrangement involving PDGFRA, PDGFRB, or FGFR1. It is important to identify these cases with PDGFRA or PDGFRB abnormalities because these patients are often responsive to TKI therapy [32].

If none of the particular cytogenetic abnormalities (listed previously) are found in a case of B or T lymphoblastic leukemia/lymphoma, the designation of "B or T lymphoblastic leukemia/lymphoma not otherwise specified" is recommended.

Philadelphia-Positive (Ph) ALL and Ph-Like ALL

Cytogenetic studies suggest that Ph chromosome occurs in 2–4% of children with ALL [33], but it is as high as 30% in adults [34]. Thus, this chromosome appears to be the single most common genetic finding in adult ALL. Moreover, the presence of the Philadelphia chromosome identifies a subgroup of ALL with a poor prognosis [35]. Most BCR-ABL1-positive childhood cases express p190 fusion protein, whereas in adults, approximately 50% of the cases express p210 kDa fusion protein (more likely in chronic granulocytic leukemia). It has been suggested that Philadelphia-positive-BCR-positive ALL cases expressing p210 may be CML in lymphoid blast crisis; BCR-negative cases expressing variant p190 may be true de novo ALL [36]. However, no clinical data have been reported to substantiate this claim. One characteristic of Philadelphia-positive ALL is the expression of both myeloid and lymphoid antigens [37]. This feature supports that the cell origin is more immature than the other B-ALL cases [38]. There are no unique morphologic or cytochemical features that distinguish Philadelphia chromosome-positive ALL from other types of ALL.

Recently in about 20% of patients with B lymphoblastic acute leukemia/lymphoma that lack the BCR-ABL1 translocation but that show similar pattern of gene expression profile to B-ALL with BCR-ABL1 which are called as B lymphoblastic leukemia/lymphoma, BCR-ABL1-like or B lymphoblastic leukemia/lymphoma, Ph-like [39]. Leukemias with these properties have poor outcome and frequently have translocations involving other receptor tyrosine kinases, or alternatively have translocations involving either the cytokine receptor-like factor 2 (CRLF2) or less commonly rearrangements of the erythropoietin receptor (EPOR) [40]. Identification of these cases has clinical importance since they are potentially amenable to thyrosine kinase inhibitor (TKI) therapy [41].

There are no unique morphologic or cytochemical features that distinguish Ph-like ALL from other types of ALL. Blasts typically have a CD19+,CD10+ phenotype. The subset of cases with CRLF2 translocations shows very high levels of surface expression of this protein by flow cytometry.

Myeloid Leukemias

AMLs result from a neoplastic transformation of a single pluripotential hematopoietic stem cell. Evidence to support this statement is morphologic [42], immunologic [43], and chromosomal [44] as well as from in vitro cell culture studies. These studies have demonstrated trilineage dysplastic features or lymphoid antigen expressions in AML, monocytic, and erythroid precursors in cultures and elaboration of colony-stimulating factors (CSFs) from monocytes.

Four major types of AML have long been recognized: acute myeloid, acute myelomonocytic, acute monocytic, and acute erythroid leukemia. Despite these subtype definitions, Auer rods are the only consistent neoplastic marker demonstrating that a blast is myeloid and of leukemic origin. Auer rods are abnormal azurophilic crystalline-like granules that represent the coalescence of primary lysosomal granules of myeloid precursors as documented by cytochemical and ultrastructural studies [45, 46]. We have demonstrated that the addition of a peroxidase or another specific stain for primary granules increases the percentage of positive blast cells that contain Auer rods from 21 to 60%. The clinical relevance of these observations is uncertain at the present time.

Classification

The minimal requirements for morphologic specification of each case of AML are well-prepared peripheral blood and bone marrow smears prepared with a Romanowsky stain such as a Wright-Giemsa stain. To prove lineage, some routine cytochemical stains can be applied. These techniques are discussed next.

A uniform reproducible subtype classification in AML is necessary for several reasons; first, it permits comparison among various therapeutic regimens from different groups and from program to program within the same institution or cooperative group. Second, it allows for the potential identification of different clinical features and laboratory aspects that may be unique to certain subtypes. Finally with the increasing sophistication of chromosomal abnormalities and gene mutations in the AML, accurate description of myeloid subsets may permit a meaningful association of nonrandom rearrangements and translocations.

Morphologic classification of AML was proposed by the FAB Cooperative Group in 1976 [8] based on the morphologic and cytochemical features of the blast cells. The original proposal was revised and expanded in 1985 [47]. In the 2008 World Health Organization (WHO) classification, and in the upcoming revision, most of the morphologic FAB subgroups are retained in the AML, NOS category because they define criteria for the diagnosis of AML across a diverse morphological spectrum [48].

Morphologic classification accepts two types of blasts: type I (agranular) blasts are myeloblasts with open chromatin, distinct nucleoli, and immature cytoplasm without granules; type II (granular) blasts are similar to type I, except that they contain up to 20 delicate cytoplasmic azurophilic granules or Auer rods, or both (Fig. 14.8). Goasguen et al. [49] distinguished another type of blasts from promyelocytes, referred to as type III blasts; these lack major characteristics of the promyelocyte, especially the Golgi zone and eccentric

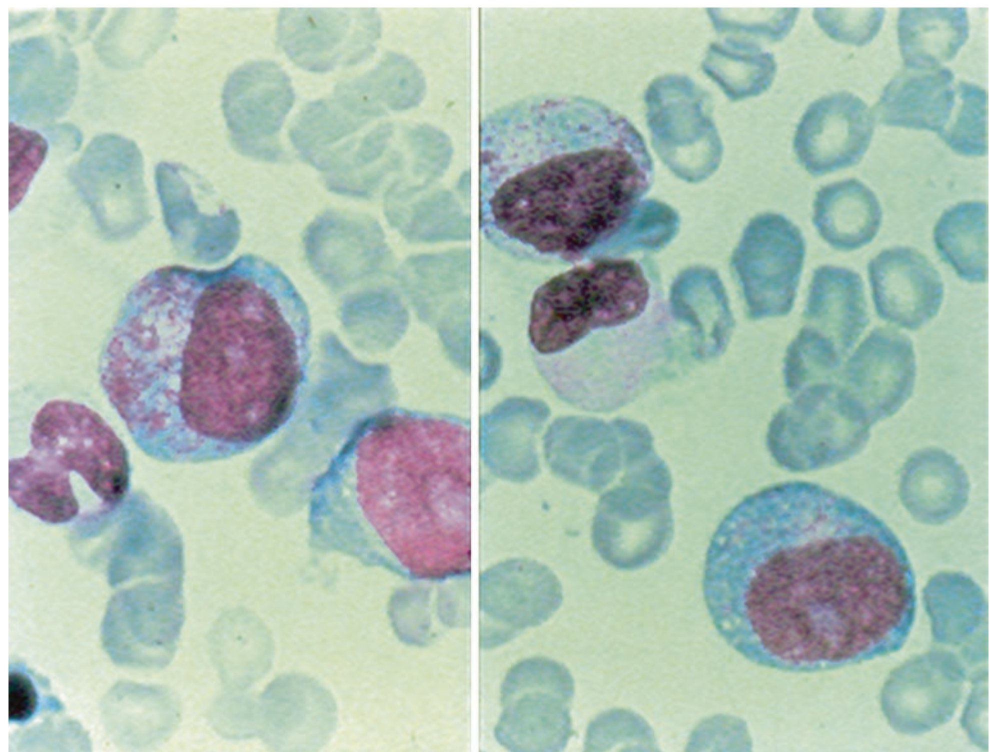

Fig. 14.8 Myeloblast morphology. *Left panel*: agranular blast and promyelocytes (note: Golgi zone); *right panel*: granular blast

nuclear location, but they have many azurophilic granules. Type III blasts are characteristically identified in AML M2 associated with t(8;21), in secondary AML and AML developing from MDS [50]. In practice, although FAB type I and type II blasts can generally be distinguished from each other it has proved difficult to distinguish FAB type II blasts from type III blasts. In addition, the enumeration of abnormal promyelocytes in MDS or AML with multilineage dysplasia remains problematic and their separation from type II and type III blasts has remained imprecise. A group of international experts in morphology has provided guidelines to assist in this regard [51].

FAB criteria identify one clinically significant type of AML, acute promyelocytic leukemia (M3); however, the remaining AML subtypes appear to be cytogenetically and immunologically heterogeneous group of diseases. AML is now classified according to the World Health Organization (WHO) Classification of Tumours of Haematopoietic and Lymphoid Tissues, which was last updated in 2008 [52]. The major categories of the current classification include AML with recurrent genetic abnormalities, AML with myelodysplasia-related changes, therapy-related AML, and AML not otherwise specified. Myeloid proliferations of Down syndrome and blastic plasmacytoid dendritic cell neoplasm are separately classified [53].

A revision of the WHO classification is under way. Changes to the section on AML with recurrent genetic abnormalities are being discussed. First, the molecular basis of inv(3)(q21q26.2) or t(3;3)(q21;q26.2) has been revisited [54]. Second, the provisional entities “AML with NPM1 mutation” and “AML with CEBPA mutation” will become entities; “AML with CEBPA mutation” will be restricted to patients with AML in whom there is a biallelic (and not a monoallelic) mutation, and third, “AML with RUNX1 mutation” [55, 56] and “AMLwith BCR-ABL1” gene [57] are being considered as provisional entities. Finally, a section on familial myeloid neoplasms, which reflects the increasing recognition of familial syndromes, is also under development [58] (Table 14.2).

In the WHO scheme, a myeloid neoplasm with 20% or more blasts in the peripheral blood or bone marrow is considered to be AML [9]. The blast count should be obtained from at least a 200-cell count of all nucleated cells in the peripheral blood and a 500-cell count of all nucleated cells in the bone marrow. In certain cases associated with specific genetic abnormalities inv (16), t(8;21), t(16;16), or t(15;17), the diagnosis of AML is made regardless of the blast count in the peripheral blood or bone marrow. Similarly, the presence of granulocytic sarcoma is diagnostic of AML even if the blast count is less than 20% in the blood or bone marrow [59].

Table 14.2 Proposed WHO revised classification of acute myeloid leukemias (AML)—2016

AML with recurrent genetic abnormalities
Acute myeloid leukemia with t(8;21)(q22;q22.1); *RUNX1-RUNX1T1*
Acute myeloid leukemia with inv(16)(p13.1q22) or t(16;16)(p13.1;q22); *CBFB-MYH11*
Acute promyelocytic leukemia with *PML-RARA*
Acute myeloid leukemia with t(9;11)(p21.3;q23.3); *KMT2A/MLL-MLLT3*
Acute myeloid leukemia with t(6;9)(p23;q34.1); *DEK-NUP214* 9869/3
Acute myeloid leukemia with inv.(3)(q21.3q26.2) or t(3;3)(q21.3;q26.2); GATA2, MECOM (EVI1)
AML (megakaryoblastic) with t(1;22)(p13.3;q13.1); RBM15-MKL1
AML with *BCR-ABL1*
AML with gene mutations
AML with mutated *NPM1*
AML with mutated *CEBPA*
AML with mutated *RUNX1*
Acute myeloid leukemia (AML) with myelodysplasia-related changes
Therapy-related myeloid neoplasms
Acute myeloid leukemia, NOS
Acute myeloid leukemia with minimal differentiation
Acute myeloid leukemia without maturation
Acute myeloid leukemia with maturation
Acute myelomonocytic leukemia
Acute monoblastic and monocytic leukemia
Acute erythroid leukemia
Acute megakaryoblastic leukemia
Acute basophilic leukemia
Acute panmyelosis with myelofibrosis
Myeloid sarcoma
Myeloid proliferations related to Down syndrome
Transient abnormal myelopoiesis associated with Down syndrome
Myeloid leukemia associated with Down syndrome
Blastic plasmacytoid dendritic cell neoplasm

Cytochemistry

Myeloperoxidase and Sudan Black B

An essential cytochemical stain is one that demonstrates the presence of MPO, an enzyme restricted to the primary granules of granulocytes and monocytes [7]. This enzyme can be identified by performing a benzidine-based peroxidase reaction or SBB staining or by diaminobenzidine reaction. The benzidine method is the best available method against which other methods should be compared. However, because of the concern for its potential carcinogenicity, it should not be adopted as the reference method [60].

The SBB reaction, a general stain for intracellular lipids, can be used in the same way. This stain is present in both primary and secondary granules. Sudanophilia and MPO activity are closely parallel [61].

Specific and Nonspecific Esterases

The chloroacetate esterase reaction can also be used to identify myeloid lineage. Although it is less sensitive than either MPO or SBB in detecting myeloid differentiation, it can be useful, when combined with ANAE in double-esterase stain, in confirming a monocytic component. Because ANAE is positive both in neutrophils and in monocytes, inhibition by NAF in monocytes should be used to identify these cells [62, 63].

Periodic Acid-Schiff Reaction

All glycogen-containing cells as well as neutrophils and granulocytes at all stages of maturation stain with this reaction. Our experience in more than 200 patients has shown that fine granular PAS reaction can be identified in all AML subtypes, ranging from 60% in AML (M1–M3) to 80% in AML M5. Moreover, the latter often demonstrates a blocklike reaction pattern characteristic of ALL (Bennett J.M., unpublished observation). The blocklike pattern of PAS reactivity is seen in erythroid precursors of acute erythroid leukemia.

Electron Microscopy

Electron microscopy (EM) is used to demonstrate either MPO or platelet peroxidase (PPO). These ultrastructural studies are useful in the confirmation of myeloid (MO) or megakaryocytic (M7) origin of blasts. MPO can be demonstrated by electron microscopy in blast cells that previously had negative results (less than 3% positive blasts) by conventional criteria. It is also useful to define the myeloid component in biphenotypic leukemias [62, 63]. In megakaryoblasts, the PPO reaction is localized exclusively on the nuclear membrane and the endoplasmic reticulum, whereas in myeloblasts it occurs in the Golgi area and cytoplasmic granules.

Immunohistochemistry

Cytospin preparations or direct marrow or peripheral blood smears as well as bone marrow paraffin sections are used to demonstrate the presence of specific lineage antigens using a secondary technique (e.g., PAP, APAAP) to show antibody fixation [22]. Its advantage includes confirmation of morphologic features, the possibility of performing retrospective studies, and in situations where peripheral blood and bone marrow smears are inadequate for study such as bone marrow fibrosis or hypocellular acute myeloid leukemia. Paraffin-reactive antibodies, i.e., CD34, MPO, CD68, CD117, CD41, CD61, glycophorin A, and factor VIII-related antigen, can successfully be used to subclassify AML [64].

Acute Myeloid Leukemias with Recurrent Cytogenetic Abnormalities

All patients morphologically diagnosed with AML must have bone marrow cytogenetic studies performed. WHO 2008 classification is recommended to incorporate recently described genetic aberrations into classification and define, biologically relevant, homogeneous entities based on not only the prognostic value of a genetic abnormality but also morphologic, clinical, and phenotypic properties [59, 65]. The contribution of cytogenetics to the classification of AML and acute myeloid leukemias with recurrent cytogenetic abnormalities are briefly discussed as follows.

Acute Myeloid Leukemia with t(8;21)(q22;q22);RUNX1-RUNX1T1

AML with t(8;21) is common in children and adults accounting for approximately 7–12% of AML overall and 20–25% of AML M2. Myeloid sarcomas may be present at presentation. Cases with t(8;21) should be diagnosed as AML regardless of a blast percentage in the bone marrow. The translocation results in a fusion product involving the RUNX1 gene (also known as core-binding factor alpha or AML1) on chromosome 21 and the RUNX1T1 (also known as ETO) gene on chromosome 8 [66]. Cases of AML with t(8;21) are associated with characteristic morphologic features [67]. Blasts are large cells with a distinctive nucleolus. The cytoplasm contains abnormal heavy azurophilic granulations (type III blasts) with tiny needlelike Auer rods (Fig. 14.9). The maturing neutrophils are commonly dysplastic. However, dysplasia of other cell lines is uncommon. Background eosinophilia is variably present. Blasts may express B-lineage antigens such as CD19, CD79a, PAX5, and TdT. These findings do not indicate the presence of mixed-phenotype leukemia in the presence of t(8;21) [68]. Acute myeloid leukemia with t(8;21) is usually associated with favorable prognosis in children and adults [69]. Mutations of KIT occur in 20–30% of cases [70]. Most published reports indicate a higher relapse rate and lower overall survival when mutated KIT is present [70, 71]. Additional mutations in ASXL2 and ASXL1 were also recently described in t(8;21) AML [72]. The presence of monosomy 7 as an additional cytogenetic abnormality may adversely impact prognosis [73]. Quantitative PCR measuring of RUNX1-RUNX1T1 transcripts is useful for monitoring minimal residual disease [74].

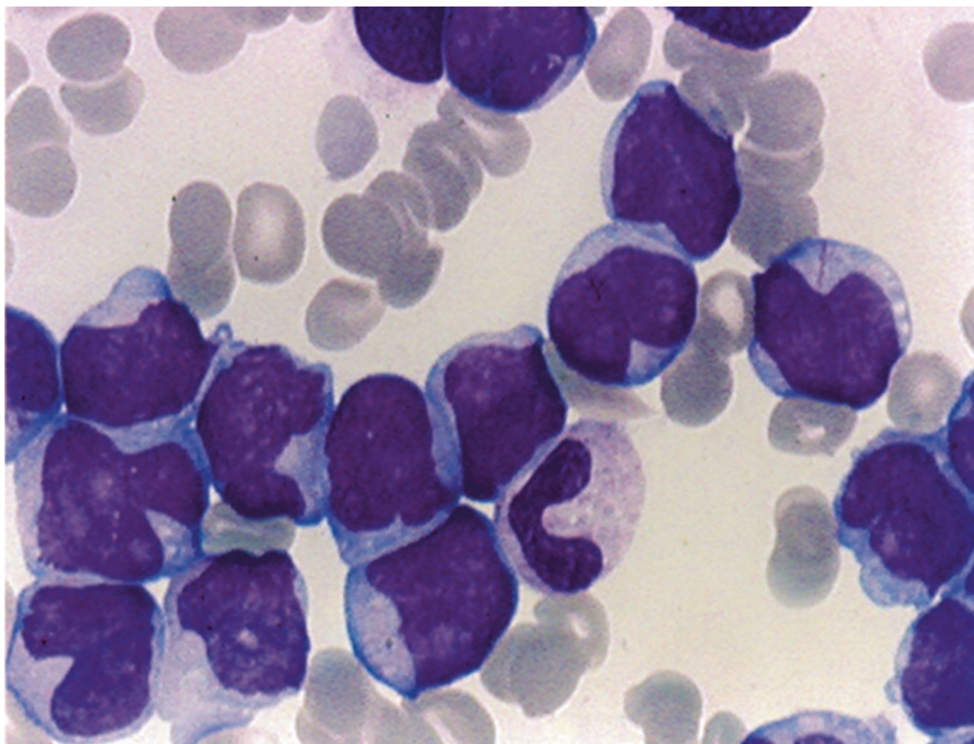

Fig. 14.9 AML –t(8;21). Note thin Auer rod in a myeloblast

Acute Promyelocytic Leukemia with t(15;17)/q22;q12, PML–RARA

APL is a distinct class of AML characterized by specific morphologic, cytogenetic, and clinical features. In most series, M3 accounts for about 10% of cases in adults and for 4–8% in children [75].

The highly specific t(15;17) translocation is present in at least 90% of all APL cases. Using molecular techniques, virtually 100% of APL cases have t(15;17). This translocation fuses the PML gene of the chromosome 15 to the retinoic acid receptor-α (RARA) gene, producing chimeric PML–RARA gene [76–78]. Cytogenetic analysis, FISH, or RT-PCR is necessary for genetic confirmation of the PML–RARA fusion. Detection of this abnormality is diagnostic of APL regardless of the blast count. The involvement of RARA at the translocation breakpoints may explain the clinical response to all-trans retinoic acid (ATRA) [79, 80].

Morphology is still highly relevant in APL diagnosis and is a very rapid modality [81]. Typical APL is characterized by the presence of hypergranular blast cells, which have the appearance of abnormal promyelocytes. The cytoplasm is dense with coarse dark-staining granules that often obscure the nucleus. The nuclear border is generally irregular and has a folded or reniform appearance (Fig. 14.10). Cells contain bundles of delicate needlelike Auer rods, giving a meshwork appearance (so-called Faggots), found in some of the leukemic blasts. These abnormal promyelocytes are strongly positive for the MPO or SBB reactions. In contrast to other AML subtypes, dysplastic features of the myeloid series associated with leukemic proliferation are not seen. In adult patients with typical APL that achieve a CR, the prognosis is better than for any category of AML.

The FAB classification recognizes a variant of APL (FAB M3v) [82], characterized by bilobate cells or by cells with reniform nucleus and cytoplasm with minimal or no granulations (Fig. 14.11). Typical M3 cells are infrequent in the bone marrow. Hypogranular promyelocytes are strongly positive for the MPO or SBB, as in typical APL. The immunophenotype of M3v is identical to that described for the typical APL (expressing of myeloid antigens in the absence of HLA-DR reactivity) [83, 84]. Special attention should be given to differentiate these cases from M4 and M5b subtypes. The distinction is important because of the well-known association of M3 with disseminated intravascular coagulation (DIC) [1]. The DIC syndrome is present equally in the M3v. In adults, the hypogranular or “microgranular” variant constitutes 25% of APL cases [85]. It may also occur in children [86]. The hematological characteristic of M3v at onset, in addition to DIC, is hyperleukocytosis (>20,000/mm^3). M3v has been associated with shorter CR and poorer overall survival, reflecting early hemorrhagic deaths [85, 86].

FLT3 mutations are common in APL with the majority being internal tandem duplication (ITD) mutations. These mutations are strongly associated with the hypogranular subtype with hyperleukocytosis [87].

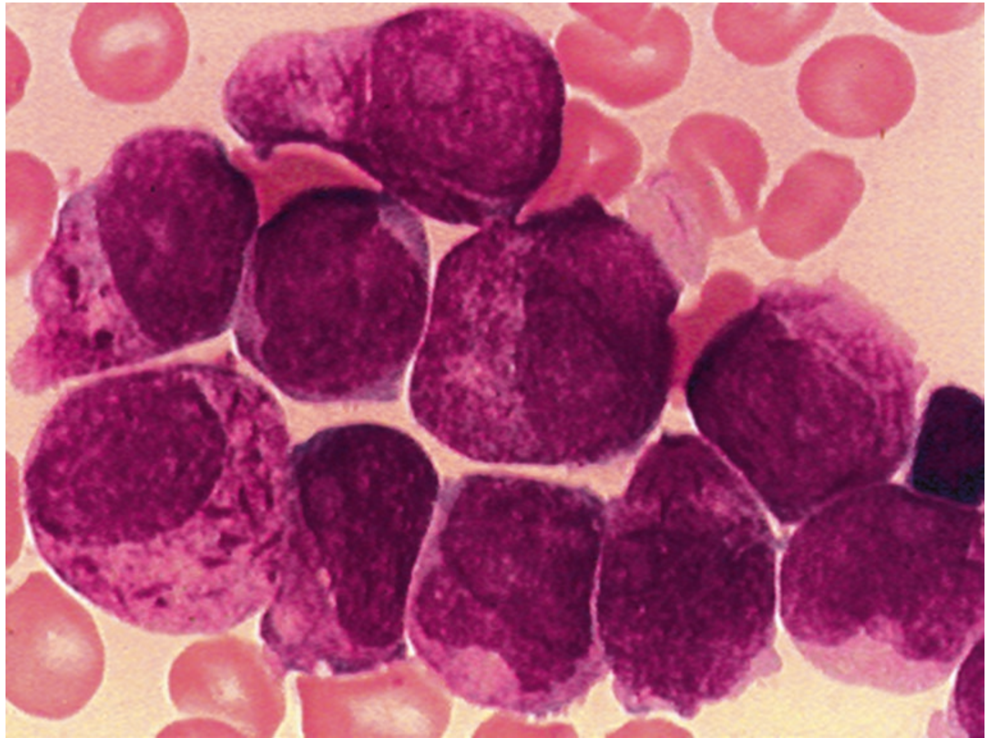

Fig. 14.10 APL (FABM3). Hypergranular promyelocytes with bundles of Auer rods

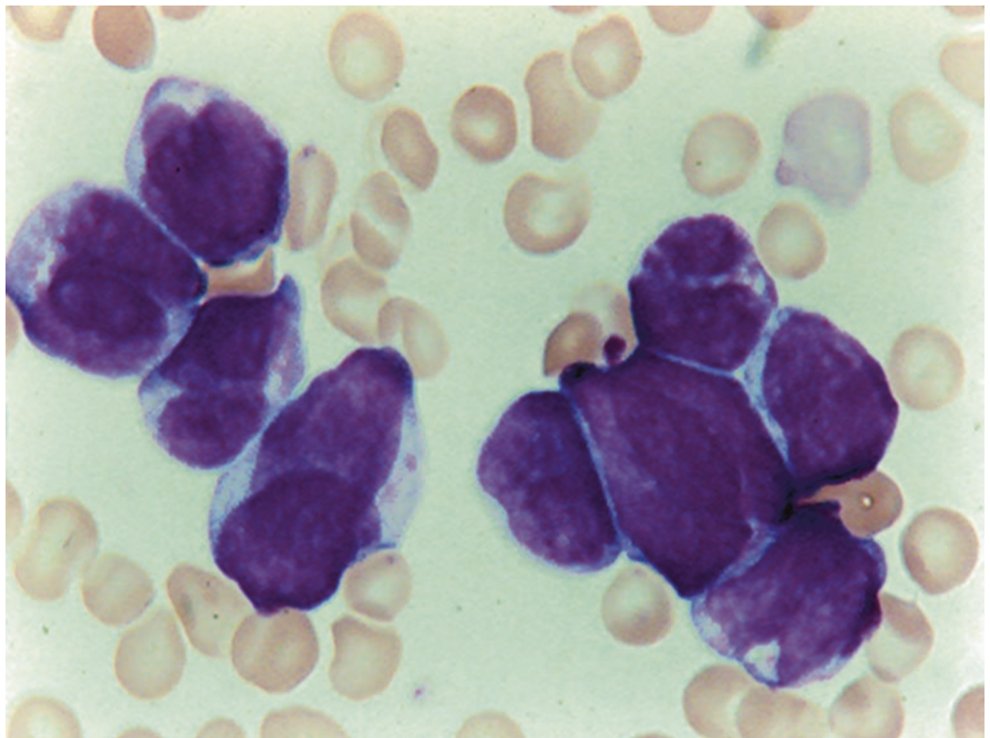

Fig. 14.11 APL variant (FABM3v). Only occasional cells have granules but prominent bilobed and reniform nuclei. WG stain

A small numbers of cases, often with morphologic features resembling APL, show variant translocations involving RARA gene on chromosome 17, but not the PML gene on chromosome 15, such as t(11;17) (ZBTB16-RARA), t(5;17)(NPM-1-RARA), and t(17;17)(STAT5B-RARA) [88]. Patients with variant RARA translocations often experience DIC. Cases with t(11;17) show morphologic differences in which Auer rods are usually absent and pelgeroid neutrophils may be seen. Importantly, some of these variant PML–RARA transcripts are associated with ATRA resistance, and some are not detected by current PCR techniques [89, 90].

Acute Myeloid Leukemia with inv(16)(p13.1q22) or t(16;16)p13.1;q22;CBFB-MYH11

AML with chromosome 16 abnormalities comprises 10% of adult and 6% of childhood AML. The inv(16) and the t(16;16) both result in the fusion of the beta-subunit of core-binding factor (CBFB) gene at 16q22 to the gene encoding smooth muscle myosin heavy chain (MYH11) at 16p13.1 [91]. The presence of this genetic abnormality is diagnostic of AML regardless of the blast count [52]. In these cases, in addition to characteristic morphologic features of acute myelomonocytic leukemia, the bone marrow shows a variable number of abnormal eosinophil components (AML-M4Eo) (Fig. 14.12). Eosinophils account for 5% or more of nonerythroid cells. The eosinophils are abnormal; in addition to the specific eosinophilic granules, they have large basophilic granules and demonstrate chloroacetate esterase and PAS (coarse granules) positivity. Often the nuclei have pseudo-Pelger features. At least 3% of the blasts show myeloperoxidase (MPO) or Sudan Black B reactivity. The monoblasts and promonocytes usually show NSE or ANAE reactivity [92]. The incidence of extramedullary disease is higher than for most types of AML, with a high incidence of CNS relapse. Patients with chromosome 16 abnormalities tend to respond to chemotherapy better, experience relatively long remissions, and have a better prognosis [69, 93]. KIT mutations are present in 30% of cases and negatively impact prognosis in older patients [70]. AML with t(8;21) or inv(16) and mutated KIT are considered as intermediate-risk AML, not favorable-risk AML [94].

Acute Myeloid Leukemia with t(9;11)(p22-q23); MLLT3-MLL

Translocations involving the MLL gene on chromosome 11q23 are seen in approximately 6% of cases of AML and secondary leukemias that occur in patients treated with topoisomerase II inhibitors [95], in patients with acute lymphoblastic leukemia, and rarely in patients with MDS [96]. However, with regard to copy number alterations (CNAs), no differences were found in the number or type of lesions between de novo and therapy-related AML with t(9;11) [97]. A number of different partners for the balanced 11q23 translocations have been identified. However, the WHO 2008 classification only recognizes t(9;11) (MLLT3-MLL) as a specific entity. This type of AML occurs at any age but is more common in children [98, 99]. Leukemic blasts containing 11q23 translocations generally have monocytic features and are subclassified as FAB M4 or M5 [100]. However, it is also detected in AML with or without maturation. Monoblasts and promonocytes show strong positive NSE reactions and often lack MPO reactivity. Extramedullary disease of the skin and gingiva and presentation with DIC have also been described.

Although pediatric cases with t(9;11) have an intermediate prognosis, leukemias associated with a different chromosome 11q23 translocations have poor prognosis [101]. Recently, within all t(11q23) AMLs, EVI1 positivity was found to be sole prognostic factor, predicting for inferior overall survival [102]. KIT or FLT3-ITD mutations are rare in AML with 11q23 translocations [59].

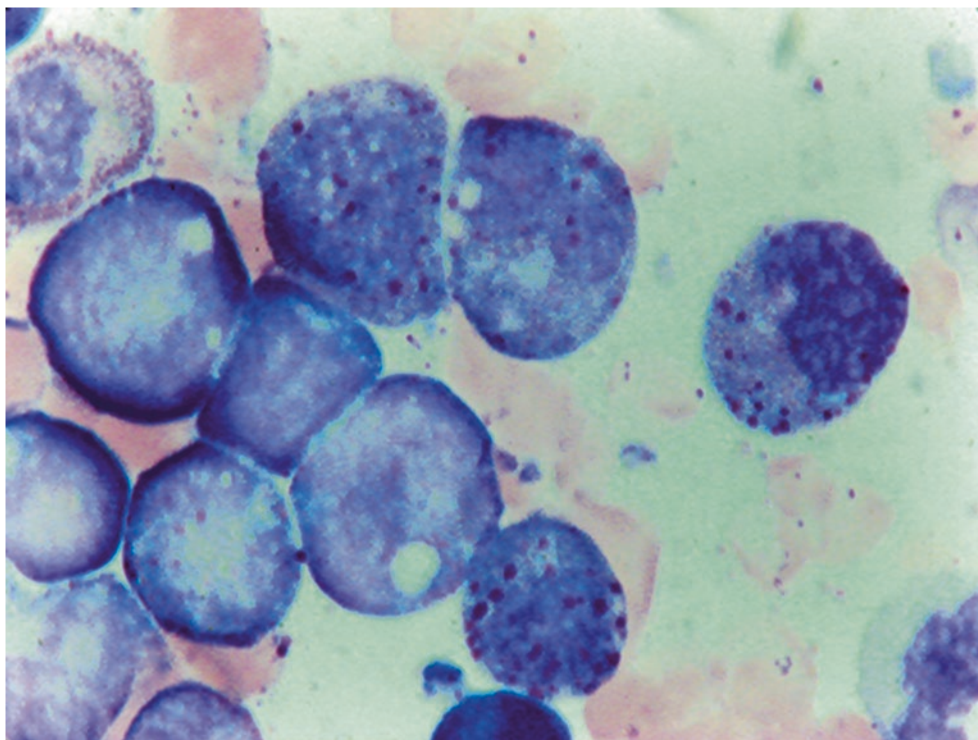

Fig. 14.12 AMML with eosinophils (FAB M4Eos). Note the magenta staining granules in the myelocyte. WG stain

Acute Myeloid Leukemia with t(6;9)(p23;q34);DEK-NUP214

The t(6;9) is detected in 0.7–1.8% of AML that occurs in both children and adults.

In this entity there are no features specific to blast cells. The blasts may show occasional Auer rods and may exhibit monocytic features. Marrow and peripheral blood basophilia defined as more than 2% is seen in half of the reported cases. Multilineage dysplasia may be evident in most of the cases [103, 104]. Blasts are MPO positive. TdT may be positive in some cases. FLT3-ITD mutations are common in this type of AML occurring in 69% of childhood and 78% of adult cases [103, 104]. AML with t(6;9)(p23;q34) represents a unique subtype of acute myeloid leukemia with a high risk of relapse, high frequency of FLT3-ITD mutations, and a specific gene expression signature including several upregulated genes involved in histone modification, and a typical HOXA/B profile, which may be a target for future therapy [105]. AML with t(6;9)(p23;q34) has poor outcome in both adults and children. However, recent smaller studies, including both adult and pediatric patients, have shown that treatment with early allogeneic hematopoietic stem cell transplantation in first complete remission may improve the outcome [106, 107].

Acute Myeloid Leukemia with inv (3)(q21q26.2) or t(3;3)(q21;q26.2);RPN1-EVI1

Various categories of 3q abnormalities in AML can be distinguished according to their genetic features. But 2008 WHO classification of hematopoietic tumors only recognizes AML with inv(3)(q21q26.2) or t(3;3)(q21;q26.2);RPN1-EVI1 as an independent clinicopathological entity, with an extremely poor prognosis. In the upcoming revised WHO classification, the molecular basis of inv(3)(q21q26.2) or t(3;3)(q21;q26.2) shows rearrangement of a GATA2 oncogenic enhancer element, rather than of the RPN1 gene, in band 3q21 with the EVI gene in band 3q26.2 [54]. High expression of the oncogene EVI1 at 3q26.2 is a poor prognostic indicator independent of 3q26 rearrangement. Deregulated expression of EVI1 is the molecular hallmark of this disease; however, when the genome-wide spectrum of cooperating mutations is elucidated it has been shown that 98% of inv(3)/t(3;3) myeloid malignancies harbor mutations in genes activating RAS/receptor tyrosine kinase (RTK) signaling pathways which may provide a target for a rational treatment strategy [108].

Acute myeloid leukemia with inv (3) or t(3;3) may represent de novo or may arise from MDS. It accounts for about 1–2% of all AML cases and occurs most commonly in adults [98, 109]. Patients typically present with anemia and sometimes thrombocytosis [110, 111]. Dysplastic hypogranular neutrophils, pseudo-Pelger cells, dysplastic thrombocytes, and naked megakaryocyte nuclei associated with or without blast cells are noted as peripheral blood changes. Bone marrow blasts may show all morphological FAB diagnoses from M0 to M7 (except M3) subtypes. MPO reactivity is often low. Multilineage dysplasia of bone marrow elements other than blast cells is a common finding. Dyserithropoesis and/or dysgranulopoiesis are frequent. Megakaryocytes may be normal or increased in number and usually have dysplastic features [111–113]. Bone marrow cellularity and fibrosis are variable.

Secondary karyotypic abnormalities including monosomy 7, 5q deletions, and complex karyotypes are present in most of the cases and associated with poor prognosis [112]. Some patients having these translocations may present less than 20% blasts in the bone marrow and should be closely monitored for development of AML.

Acute Myeloid Leukemia (Megakaryoblastic) with t(1;22)(p13;q13); RBM15-MKL1

Acute megakaryoblastic leukemia (AMKL) with t(1;22) is a rare form of AML and restricted to infants and children younger than 3 years. It commonly occurs in infants without Down syndrome (DS) [52, 114]. Non-DS AMKL is characterized by chimeric oncogenes consisting of genes known to play a role in normal hematopoiesis [115, 116]. The median age at diagnosis is 4 months. Majority of cases present with marked hepatosplenomegaly and/or osteolytic skeletal lesions with or without bone marrow involvement [117]. Patients also have anemia and thrombocytopenia with a moderately elevated white blood cell count. The morphology of the blast cells reveals that cells are very pleomorphic that may vary from very small with dense chromatin to somewhat larger with a fine reticulated nuclear chromatin and prominent nucleoli. Cytoplasmic blebs or actual platelet shedding may be found surrounding some blasts. The blasts are MPO or SBB negative. Micromegakaryocytes are common, but dysplastic features of erythroid and granulocytic cells are not present. Bone marrow biopsy may show many clusters of micromegakaryoblasts, as well as more mature megakaryoblasts (Fig. 14.13). This is associated with an increase in reticulin formation and a corresponding decrease in normal hematopoietic precursors. Patients with t(1;22) and less than 20% blasts on bone marrow aspiration should be correlated with the bone marrow biopsy. The presence of myeloid sarcoma is diagnostic of AML regardless of the marrow blast count. Megakaryoblasts express one or more of the platelet glycoproteins (CD41 and CD61). Cytoplasmic expression of these markers is more specific and sensitive than surface staining. The myeloid-associated markers CD13 and CD33 may be positive. CD36 is characteristically positive. CD34,

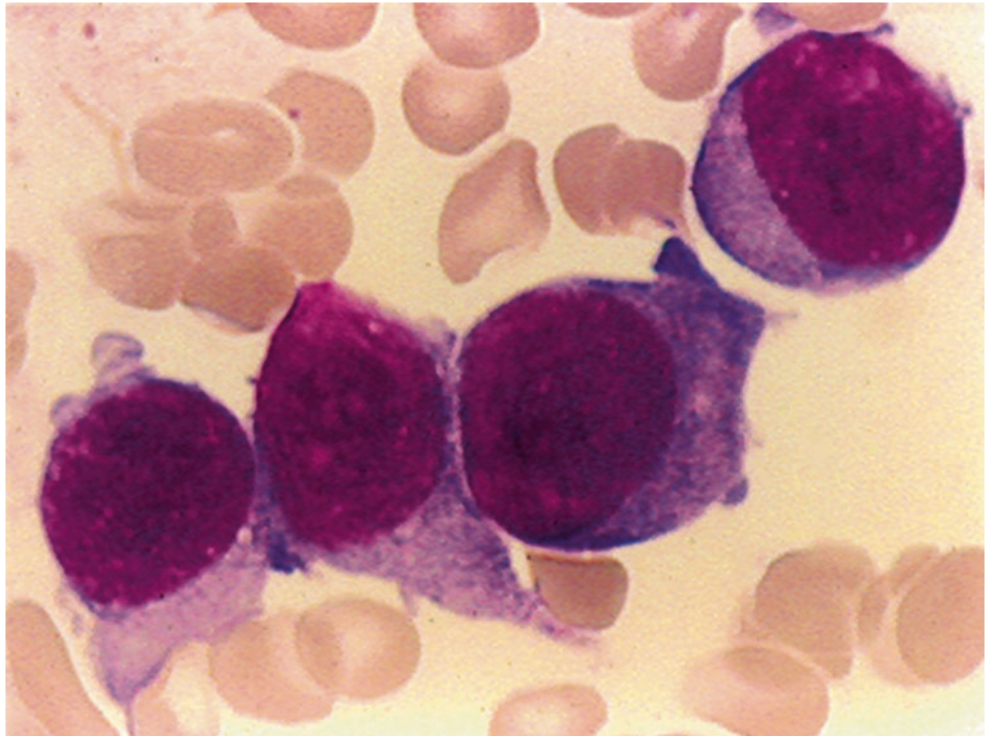

Fig. 14.13 Acute megakaryocytic leukemia. (FABM7). Large blasts with cytoplasmic projections. Immunostain for CD41 was positive. WG stain

MPO, HLA-DR, and TdT are negative [49]. The outcome of non-DS AMKL is generally poor, with lower event-free survival even in the face of intensified treatment [118]. Monitoring of MRD using RBM15-MKL1 fusion transcript would be useful in the treatment of AMKL with t(1 ; 22)(p13; q13) [119].

AML with Gene Mutations

In addition to translocation and inversions, specific gene mutations also occur in AML. Alone or in combination NPM1, FLT3-ITD, CEBPA, and KIT mutations have been reported in AML patients with a normal karyotype. However, they may also be seen in patients with translocation and inversions as well [119]. Recent studies have also shown that the mutations of ASXL1 and TP53 as well as RUNX1 have consistently been associated with an inferior outcome and they will be included in these recommendations [65, 120]. In the 2008 WHO classification [52], mutations of NPM1, FLT3-ITD, and CEBPA are listed among the most common recurrently mutated genes, and those with the NPM1 or CEBPA mutation are designated as provisional entities; in revised upcoming edition they will become entities. On the other hand, AML with CEBPA mutation will be restricted to patients with AML in whom there is a biallelic (and not a monoallelic) mutation, because only that form of AML defines a clinicopathologic entity that is associated with a favorable prognosis [121]. Finally, "AML with RUNX1 mutation" [56] is being considered as provisional entity on the basis of their characteristic clinicopathologic features. FLT3-ITD gene mutations may occur with any type of AML and MDS (20–40% of cases) but is more common in AML with t(15;17) and AML with a normal karyotype and their presence is associated with an adverse outcome [122, 123]. KIT mutations have prognostic significance in cases of AML with t(8;21), inv(16), and t(16;16) (core-binding factor leukemias), in which they are associated with a poor prognosis [70]. AML with mutated NPM1 is found in 50% of adult and 20% of pediatric AML patients with a normal karyotype [124, 125]. AML with mutated NPM1 in the absence of a FLT1-ITD mutation has a favorable prognosis [124, 126]. In adults, most of the NPM1-mutated cases show monocytic differentiation. Along with molecular techniques, paraffin sections are also used to show aberrant cytoplasmic expression of NPM by immunohistochemistry [125]. Around 10% of AML cases with normal karyotype carry the CEBPA mutation. Most CEBPA-mutated cases are double mutated (biallelic); only a few cases of AML cases with normal karyotype carry a singly mutated CEBPA [127]. There are no distinctive morphologic features of AML with biallelic mutations of CEBPA, but the vast majority of cases have features of either AML without or with maturation [127, 128]. Multilineage dysplasia is reported in 26% of cases with no adverse prognostic significance [129]. *RUNX1* mutations have been identified in a substantial proportion of AML patients with normal karyotype [55, 56] as well as in myelodysplastic syndromes [130]. AML with RUNX1 mutations

are often associated with undifferentiated morphology (M0). RUNX1 mutations are frequently associated with ASXL1 mutations; however they never coexisted with NPM1 and CEBPA mutations [56]. Since RUNX1 mutations are associated with poor clinical outcome in both younger and older patients treated with intensive induction chemotherapy [55] patients harboring RUNX1 mutations warrant novel therapies and/or early alloSCT.

Acute Myeloid Leukemia with Myelodysplastic Related Changes

Patients are assigned to "AML with myelodysplasia-related changes" if they have 20% or more blasts in the blood or marrow and (1) arise from previous myelodysplastic syndrome (MDS) or a myelodysplastic/myeloproliferative (MDS/MPN) neoplasm, (2) have specific MDS-related cytogenetic abnormalities, and/or (3) exhibit multilineage dysplasia (50% or more of the cells in two or more myeloid lineages) [49]. Patients should not have a history of prior cytotoxic or radiation therapy for an unrelated disease. The specific genetic abnormalities of AML with recurrent genetic abnormalities are absent.

This category of AML occurs mainly in elderly patients and is rare in children [131]. Although the definition of multilineage dysplasia is variable in the literature, this category appears to represent 24−35% of all cases of AML [132, 133]. Myeloid sarcomas may be present at presentation and should be diagnosed as AML regardless of the bone marrow blast percentage [52].

To classify an AML as having myelodysplasia-related changes based on morphology, dysplasia must be present in at least 50% of the cells in at least two myeloid cell lines. Morphologic dysplasias are characterized by neutrophils with hypogranular cytoplasm or hyposegmented nuclei; erythroblasts with megaloblastoid changes, nuclear irregularity, multinuclearity, and ringed sideroblasts; megakaryocytes with nonlobated or multiple nuclei; and micromegakaryocytes. Dysmegakaryopoiesis may be more easily recognized in paraffin sections [132, 134]. Some cases do not meet the criteria for a morphologic diagnosis. However, they are diagnosed as AML with myelodysplastic related changes by the detection of MDS-related cytogenetic abnormalities and/or by a prior history of MDS or MDS/MPN. Chromosome abnormalities are similar to those found in MDS and often involve gain or loss of major segments of certain chromosomes with complex karyotypes, −7/del(7q) and del(5q), and unbalanced translocations involving 5q being most common [93, 135]. Cases of AML with multilineage dysplasia may carry NPM1 and/or FLT3 mutations, or biallelic mutations of CEBPA [136]. Most NPM1-mutated or CEBPA double-mutated cases would be expected to have a normal karyotype and no history of prior MDS [137]. Prognosis of these cases is similar to cases without multilineage dysplasia [129]. Therefore, such cases are now considered as part of the respective entities of AML with mutated NPM1 or AML with double-mutated CEBPA and not as AML with myelodysplasia-related changes. Other gene mutations, such as U2AF1, ASXL1, and TP53, are fairly common in AML with myelodysplasia-related changes [9, 138]. Blasts often express panmyeloid markers (CD13, CD33), but aberrantly high or low expression is common. There is frequent aberrant expression of CD56 and/or CD7 [139].

The principal differential diagnoses are MDS with excess blasts (MD-EB), acute erythroid leukemia, acute megakaryoblastic leukemia, and other categories of AML, not otherwise specified (NOS). Careful blast cell counts, adherence to the diagnostic criteria for morphological dysplasia, and evaluation for MDS-related cytogenetic abnormalities should resolve most cases, with this category having priority over the purely morphological categories of AML, NOS. For example, a case with ≥20% total BM myeloblasts, multilineage dysplasia, ≥50% BM erythroid precursors, and monosomy 7 should be considered as AML with myelodysplasia-related changes rather than acute erythroid leukemia. Similarly, a case with 20% or more BM megakaryoblasts and multilineage dysplasia would be considered AML with myelodysplasia-related changes if AML with t(1;22)(p13.3;q13.1) and myeloid neoplasms of Down syndrome are excluded.

Although AML with multilineage dysplasia is generally associated with a poor prognosis, several studies have not found morphology to be a significant parameter when using multivariate analysis that also incorporates the results of cytogenetic analysis, high-risk cytogenetic abnormalities being more significantly associated with prognosis [136]; however, the presence of multilineage dysplasia in the absence of prior MDS or an MDS-related cytogenetic abnormality appears to remain to be a significantly poor prognostic indicator in adults [140, 141].

Therapy-Related Myeloid Neoplasms

This category includes therapy-related AML, MDS, and MDS/MPN occurring as late complication of cytotoxic and/or radiation therapy administered for a neoplastic or non-neoplastic disorder [142]. Therapy-related myeloid neoplasms account for 10–20% of all cases of AML, MDS, and MDS/MPN [143].

Two subsets of therapy-related neoplasms are recognized. The longer latency cases (5–10 years after therapy) are associated with alkylating agents and ionizing radiation. This category is commonly associated with chromosomal losses, often of −5,−7 in a setting of complex karyotype and mutations or loss of TP53 [144, 145]. Shorter latency cases may arise 1–5 years after therapy and comprise 20–30% of

patients. These cases are usually associated with topoisomerase II inhibitor therapy and majority of these cases are associated with recurrent balanced chromosomal translocations that frequently involve 11q23 (MLL orKMT2A) or 21q22.1 (RUNX1) and have morphology resembling de novo acute leukemia associated with these same chromosomal abnormalities [146]. On the other hand, division of patients according to the type of the therapy is not practical since most patients have received polychemotherapy that includes both classes of drugs [147]. The bone marrow may be hypercellular, normocellular, or hypocellular and may have been associated with fibrosis. Blast counts are variable; approximately half of the t-MDS patients will have less than 5% blasts but often exhibit poor-risk cytogenetics [148, 149]. Dysgranulopoiesis and dyserythropoiesis are present in most cases. Megakaryocytes vary in number but the majority of cases show dysplastic megakaryocytes with mono- or hypolobated nuclei. In 20–30% of cases the first manifestation of a therapy-related myeloid neoplasm is overt acute leukemia without a preceding myelodysplastic phase [146–148]. Many of these cases have monoblastic or myelomonocytic morphology. Some cases may have karyotypic and morphologic changes identical to de novo AML with recurring cytogenetic abnormalities, including t-APL with RARA-PML. Such cases should be designated as t-AML with the appropriate cytogenetic abnormality indicated [150]. The prognosis of t-MN is generally poor although it is strongly influenced by the associated karyotypic abnormality as well as the comorbidity of the underlying malignancy or illness for which the cytotoxic therapy was given [151].

Acute Myeloid Leukemia, Not Otherwise Specified

Acute myeloid leukemia, not otherwise specified (AML, NOS), encompasses those cases that do not fulfill the criteria for any of the other previously described AML categories. AML, NOS accounts for 25–30% of all cases. The subgroups of AML, NOS are not prognostically significant [132, 152] when AML with mutated *NPM1* and double-mutated *CEBPA* are removed [48]. Mutation analysis and cytogenetic studies are essential before a case can be placed into this category.

The primary basis for subclassification within AML, NOS category is the morphological and cytochemical/immunophenotypic features of the leukemic cells that indicate the major lineages involved and their degree of maturation. The defining criterion for AML is the presence of 20% or more myeloblasts in the peripheral blood (PB) or bone marrow (BM); the promonocytes in AML with monocytic differentiation are considered blast equivalents. The classification of pure erythroid leukemia is unique and is based on the percentage of abnormal, immature erythroblasts. The previous category of erythroid/myeloid type of erythroleukemia has been eliminated from AML, NOS; cases with a myeloblast count of less than 20% of total BM and PB cells are considered as myelodysplastic syndrome, while cases with greater than 20% myeloblasts will continue to be classified according to standard AML criteria.

Acute Myeloid Leukemia with Minimal Differentiation (M0)

AML M0 is a rare form of acute myeloid leukemia consisting approximately less than 5% of AMLs and may occur at any age. Morphologically, blasts are large with open chromatin and prominent single or multiple nucleoli. The N/C ratio is low. Cytoplasm is moderately basophilic. Azurophilic granules or Auer rods are not seen (Fig. 14.2). MPO- or SBB-positive blasts are less than 3% by light microscopy. Without immunophenotyping, AML M0 may be misdiagnosed as ALL based on the negative cytochemical reactions. So, it is important to stress that a diagnosis of AML M0 cannot be made on morphologic grounds alone and that always requires confirmation by immunologic techniques. Blast cells usually expressed CD13 and CD117 as well as early, hematopoietic associated antigens (CD34, HLA-DR, CD38), while expression of CD33 is found in approximately 60% of cases. Since CD13 antigen is expressed in the cytoplasm of myeloblasts earlier than on the surface, CD13 should be tested by immunohistochemistry whenever the flow cytometry on the cell suspensions is negative. Moreover lymphoid differentiation antigens should be absent, except for CD7 and TdT. TdT is positive in approximately 50% of cases and has been suggested to be of favorable prognostic significance [153]. Patients with the AML M0 subtype have a poorer response to combination therapy.

Acute Myeloblastic Leukemia with Minimal Differentiation (M1)

Poorly differentiated myeloblasts are the predominant nonerythroid cell type (type I and type II). Auer rods may be present and consistent with the diagnosis (Fig. 14.14). More than 3% of these blast cells are MPO or SBB positive by conventional cytochemistry (Fig. 14.3). The low percentage of MPO-positive (3–10%) M1 cases may constitute up to 25% of all M1 cases [154]. In such cases, M1 should be differentiated from ALL L2, acute megakaryoblastic leukemia, and acute monoblastic leukemia without differentiation (M5a). Cytochemical stains and immunophenotyping using a classic panel (MPO, CD13, CD33, CD15, CD34, CD117) to confirm the myeloid nature of blasts are necessary for the differential diagnosis. Blasts are negative for B- and T-associated

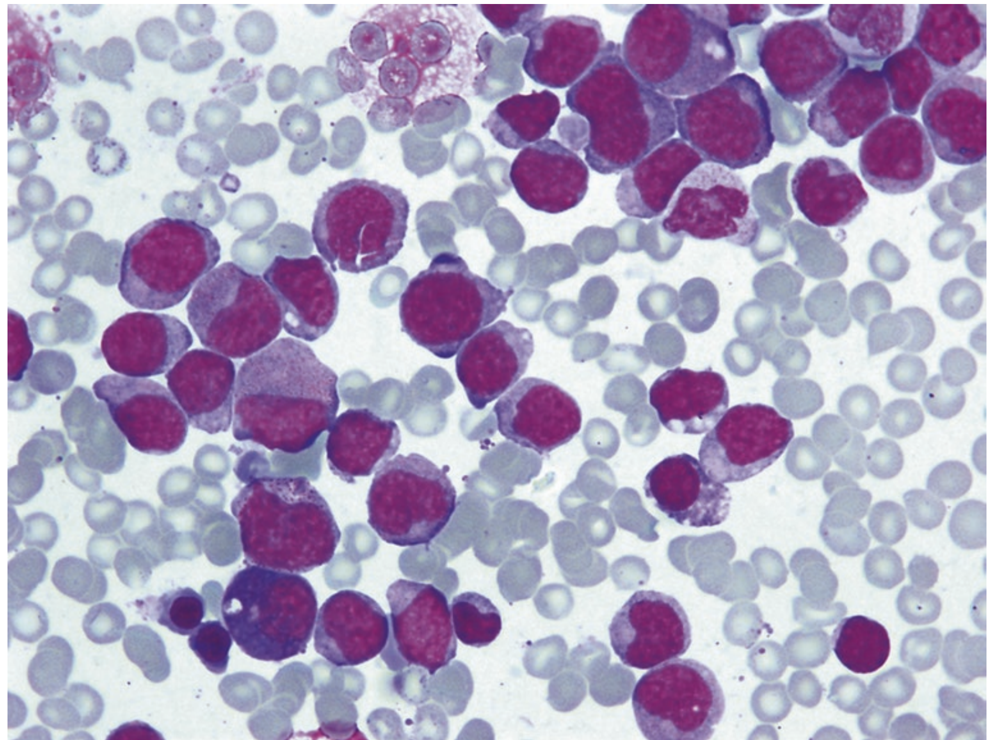

Fig. 14.14 AML (FABM1). Myeloblasts with <10% maturation of granulocytes. WG stain

cytoplasmic lymphoid markers: cCD3, cCD79a, and cCD22. CD7 is found in ~30% of cases, while expression of other lymphoid-associated membrane markers such as CD2, CD4, CD19, and CD56 has been described in 10–20% of cases.

Acute Myeloblastic Leukemia with Maturation (M2)

Acute myeloid leukemia with maturation is characterized by the presence of ≥20% blasts in the BM or PB and evidence of significant differentiation in all cells beyond the promyelocyte state (Fig. 14.15). Granular and agranular blasts are present. Auer rods are frequently seen. Monocytic precursors cannot exceed 20%. Increased numbers of eosinophilic precursors may be seen but they do not exhibit the cytological or cytochemical abnormalities characteristic of the abnormal eosinophils in acute myelomonocytic leukemia associated with inv(16)(p13.1q22) or t(16;16)(p13.1;q22). Variable degrees of dysplasia as pseudo-Pelger-Hüet cells and hypogranular neutrophils are frequently present, but no more than 50% of cells in two lineages are dysplastic. The MPO and SBB reactions are strongly positive. However, cases with a partial MPO deficiency in the granulocytic precursors and mature granulocytes have been reported [155]. Basophils are sometimes increased in this rare form and, therefore, this type of leukemia must be separated from CML in blast crisis by appropriate cytogenetic studies [156].

Leukemic blasts in AML with maturation usually express one or more of the myeloid-associated antigens, CD13, CD33, CD65, CD11b, and CD15. Early stem cell antigens like HLA-DR, CD34, and/or CD117 are also expressed. CD7 is expressed in 20–30% of cases.

Acute Myelomonocytic Leukemia (M4)

Acute myelomonocytic leukemia comprises 5–10% of cases of AML. It occurs in all age groups but is more common in older individuals; the median age is 50 years. Acute myelomonocytic leukemia is morphologically characterized by the proliferation of both neutrophil and monocyte precursors (Fig. 14.16). The PB or BM has ≥20% blasts including promonocytes; neutrophils, monocytes, and their precursors each comprise at least 20% of BM cells. This arbitrary minimal limit of 20% monocytes and their precursors distinguishes acute myelomonocytic leukemia from cases of AML with or without maturation in which some monocytes may be present. The PB typically shows an increase in monocytes, which are often more mature than those in the BM.

The monoblasts are large cells, with abundant cytoplasm basophilic cytoplasm, and have round nuclei with delicate lacy chromatin and one or more large prominent nucleoli. Promonocytes have a more irregular and delicately convoluted nuclear configuration; the cytoplasm is usually less basophilic and sometimes more obviously granulated. Monocytes and promonocytes may not always be readily distinguishable from maturing myeloid cells in routinely stained BM smears. In this regard cytochemical stains are necessary

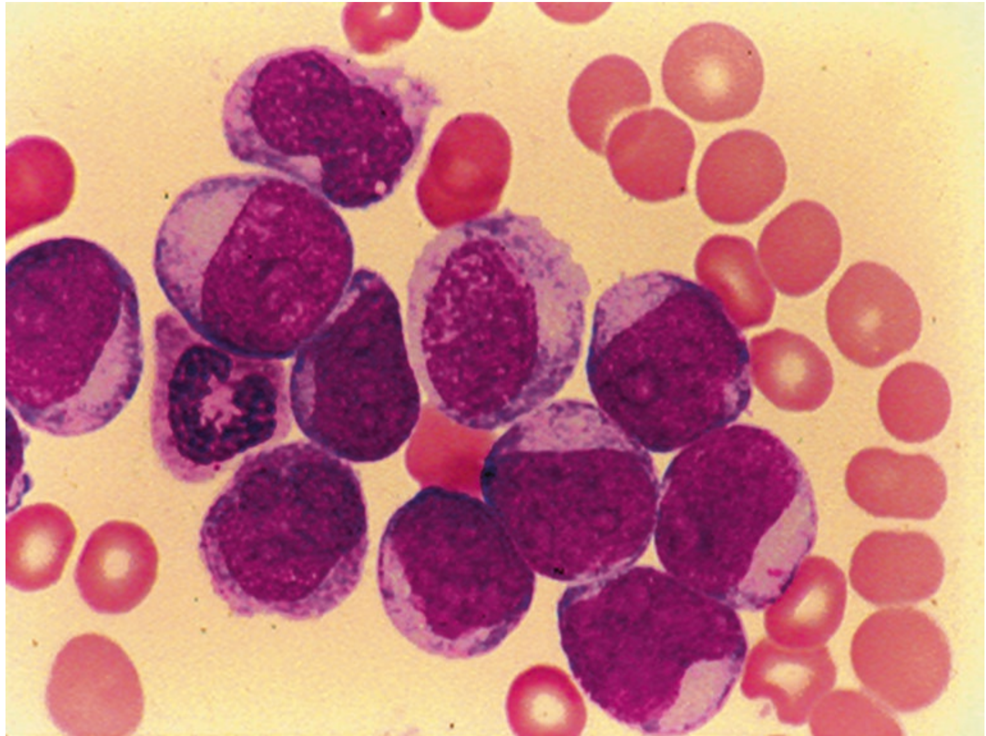

Fig. 14.15 AML (FABM2). Blasts with significant maturation of granulocytes at the promyelocyte and beyond. A single cell has an Auer rod. WG stain

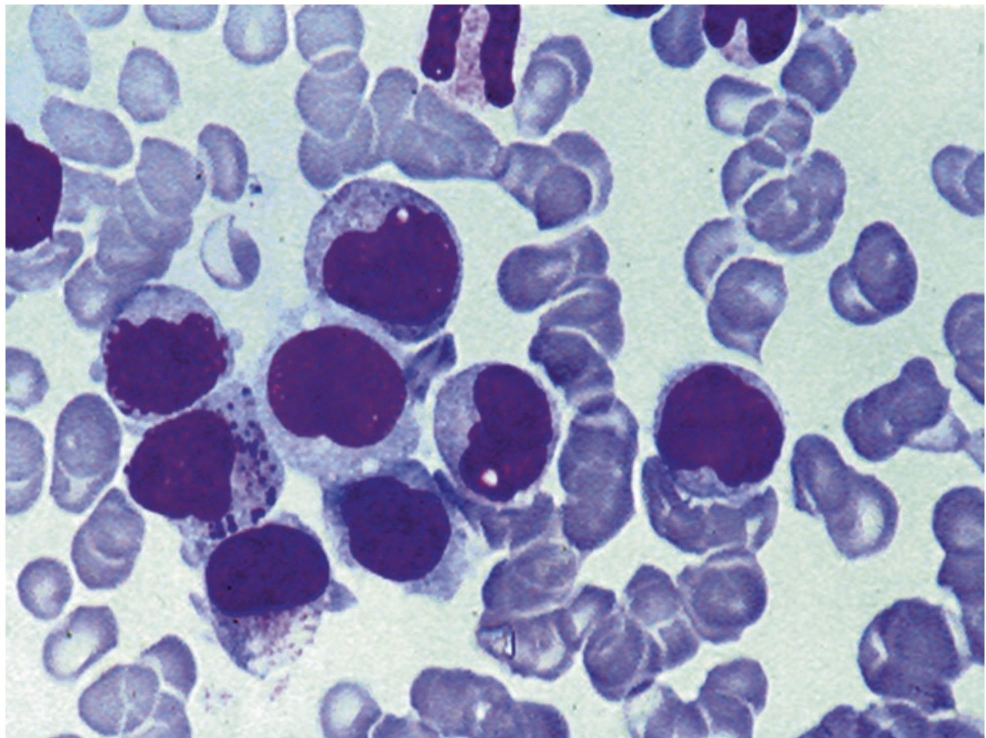

Fig. 14.16 AMML (FAB M4). Both myeloblasts and monocytic precursors are present. WG stain

for confirming the diagnosis. The monoblasts, promonocytes, and monocytes are typically nonspecific esterase (NSE) positive, although in some cases reactivity may be weak or absent. If the cells meet morphologic criteria for monocytes, absence of NSE does not exclude the diagnosis. Double staining for NSE and CAE or MPO may show dual-positive cells (Fig. 14.17). At least 3% of the blasts should show MPO positivity. Monocytic component can be identified by immunophenotyping using CD14, CD11c, CD11b, and CD68 in conjunction with other granulocyte-restricted antibodies.

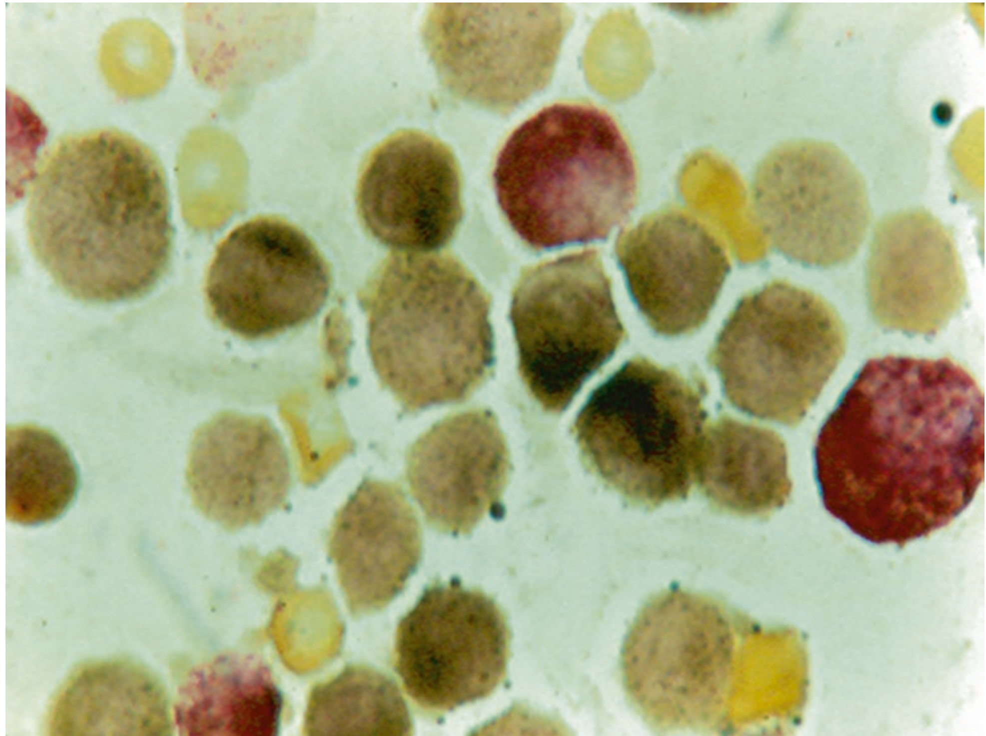

Fig. 14.17 Double-esterase reaction. *Black* product in monocytes and *brick red* color in granulocytes

Dysplastic features involving granulocytic, erythroid, and megakaryocytic lineages can be identified in approximately 20% of patients. The major differential diagnoses include AML with maturation, acute monocytic leukemia, and chronic myelomonocytic leukemia. The differential diagnosis with chronic myelomonocytic leukemia is critical and relies on the proper identification of promonocytes.

Acute Monoblastic and Monocytic Leukemia (M5a and M5b)

Acute monoblastic and acute monocytic leukemias both account for about 10% of the AML. They are clonally expressed in cells committed to differentiation to monocytic pathway [157]. The PB or BM has ≥20% blasts (including promonocytes) and in which 80% or more of the leukemic cells are of monocytic lineage including monoblasts, promonocytes, and monocytes; a minor neutrophil component, <20%, may be present.

Acute monoblastic leukemia is frequently observed in children; it can be confused with the Burkitt lymphoma/leukemia. In the bone marrow, 80% or more of all monocytic cells are monoblasts. Blast cells display abundant deep basophilic cytoplasm, which is often vacuolated and has no or few azurophilic granules and no Auer rods. The nuclei are round to oval, with one or more prominent nucleoli (Fig. 14.18). Since the peroxidase reaction may be negative in 40–50% of such cases and the PAS reaction is often strongly positive with a blocklike pattern, it is very useful to use nonspecific esterase (NSE) staining differential diagnosis. This stain will be strongly positive in more than 90% of cases. Occasionally, the SBB reaction will be positive in the absence of a peroxidase reaction. Cells with monocytic differentiation can express at least two markers characteristic of monocyte differentiation such as CD14, CD4, CD11B, CD64, CD68, and CD36 on the cell surface.

Acute monocytic leukemia is defined by the presence of 20% or more of abnormal cells being (promonocytes) with twisted or folded nuclei, gray-blue cytoplasm, and scattered azurophilic granules (Fig. 14.19). Rarely, a few cells will contain Auer rods. The percentage of mature monocytes is often much higher in the blood than in the bone marrow.

Extramedullary masses (monocytic sarcomas), cutaneous or gingival infiltration, and CNS involvement are common. MPO and CEA are typically negative, but CD68 and CD168 are often positive in extramedullary myeloid (monoblastic) sarcomas. Hemophagocytosis may be observed and is often associated with myeloid-associated, nonspecific cytogenetic abnormalities such as t(8;16)(p11.2;p13.3) [158].

The differential diagnosis of acute monoblastic leukemia includes AML without maturation, AML with minimal differentiation, and acute megakaryoblastic leukemia. Extramedullary myeloid (monoblastic) sarcoma may be confused with malignant lymphoma or soft-tissue sarcomas. However, they are readily distinguished by immunophenotypic analysis and cytochemistry. The differential

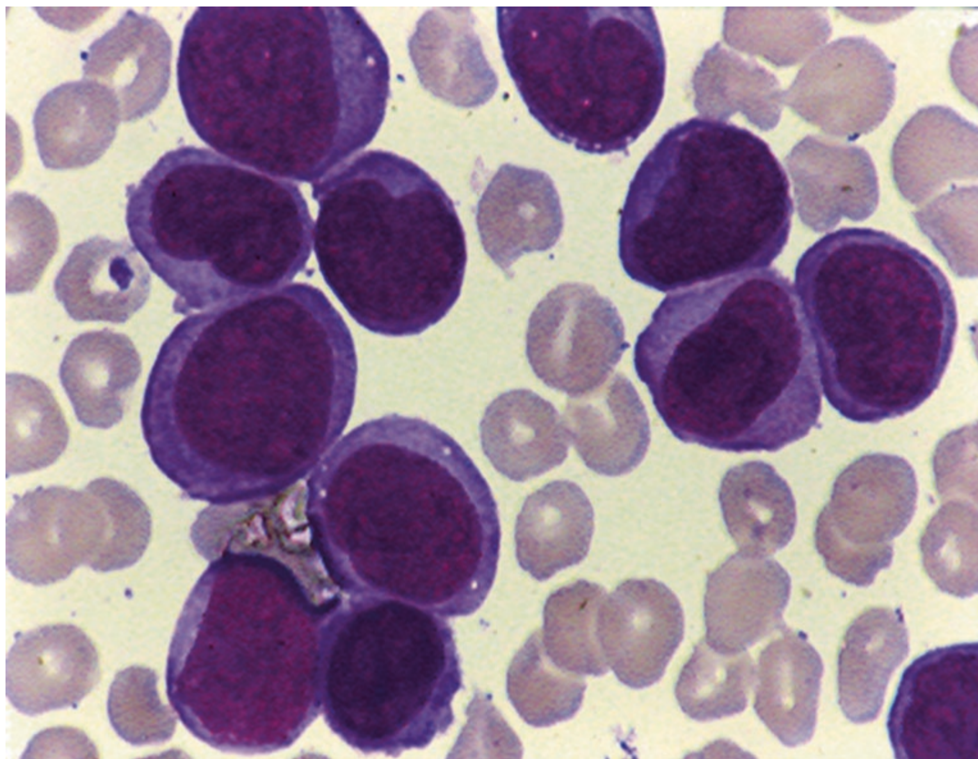

Fig. 14.18 AMOL (acute monocytic leukemia) (FABM5A). Large blasts with nuclear indentation without granules. WG stain

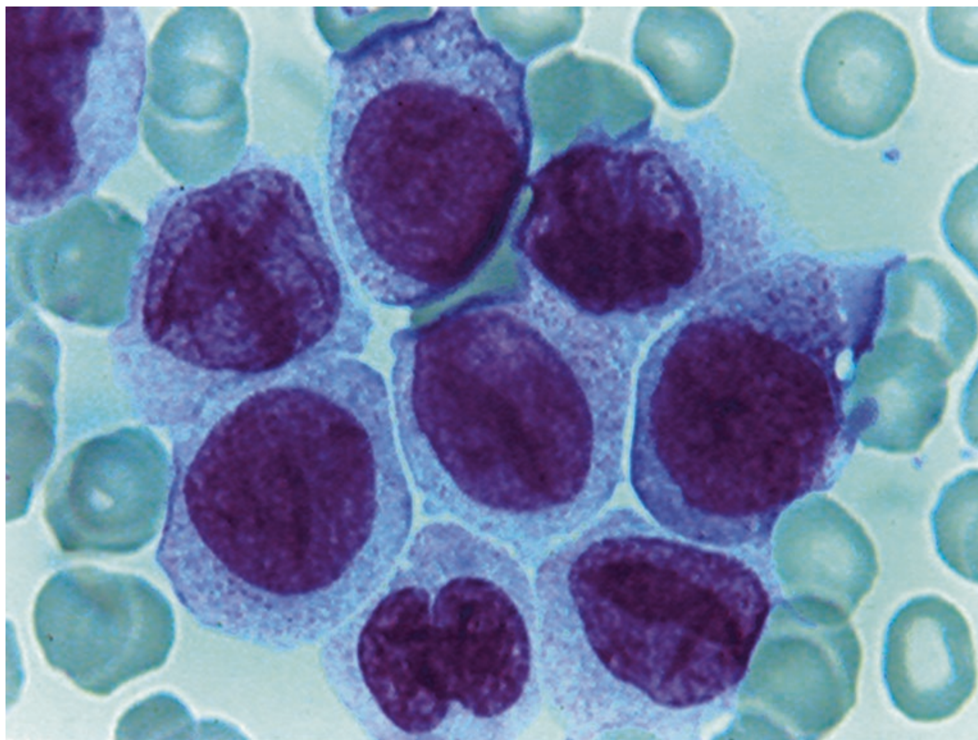

Fig. 14.19 AMOL (FABM5B). Promonocytes are apparent with occ. Azurophil granules. WG stain

diagnosis of acute monocytic leukemia includes chronic myelomonocytic leukemia, acute myelomonocytic leukemia, and microgranular acute promyelocytic leukemia (APL). These can be distinguished by careful examination of well-stained smears and by cytochemistry. The differential diagnosis with chronic myelomonocytic leukemia is critical and relies on the proper identification of promonocytes and their inclusion as blast equivalents.

Acute Erythroid Leukemia (M6)

Pure erythroid leukemia is extremely rare and defined as a neoplastic proliferation of immature (or more mature) cells committed exclusively to the erythroid lineage (80% of total nucleated cells) with no evidence of a significant myeloblastic component [159, 160]. It can occur at any age, including in childhood.

Cases previously classified as the erythroid/myeloid subtype of erythroleukemia (Fig. 14.20), based on counting myeloblasts as a percentage of nonerythroid cells when erythroid cells comprised ≥50% of marrow cells, are now classified based on the total bone marrow or peripheral blood blast cell count. Such cases are classified as myelodysplastic syndromes (usually MDS with excess blasts) when the blast count is <20% of all cells and usually as AML with myelodysplasia-related changes when blasts are ≥20% of marrow or blood cells, irrespective of the erythroid precursor cell count.

Pure erythroid leukemia may occur as a de novo disease, but more frequently occurs as progression of a prior myelodysplastic syndrome or as therapy-related disease [161, 162]. In the latter setting, the case should be diagnosed as a therapy-related myeloid neoplasm.

Morphologically, pure erythroid leukemia is characterized by the presence of medium-sized to large proerythroblasts, with round nuclei, fine chromatin, and one or more nucleoli; the cytoplasm is deeply basophilic and agranular and frequently contains PAS-positive vacuoles (Fig. 14.21). Occasionally the blasts are smaller with scanty cytoplasm and can resemble the lymphoblasts of ALL. The cells are negative for MPO and SBB; they show reactivity with α-naphthyl acetate esterase, acid phosphatase, and blocklike PAS positivity. Pure erythroid leukemia without morphologic evidence of erythroid maturation is difficult to distinguish from megakaryoblastic leukemia and ALL. Moreover, differentiation of the more differentiated forms of pure erythroid leukemia from megaloblastic anemia is difficult.

Acute Megakaryoblastic Leukemia (M7)

Acute megakaryoblastic leukemia (AMkL) in this category is probably an uncommon disease since it would not include cases with t(1;22), AML with inv(3) or t(3;3), AML of Down syndrome, and cases meeting the criteria for AML with myelodysplasia-related changes. The WHO defines AMkL as 20% or more blasts of which at least 50% are of megakaryocyte lineage. The morphologic and immunophenotypic features are those of the megakaryoblasts (Fig. 14.13) described in the aforementioned entities. Cytochemical stains for SBB, CAE, and MPO are consistently negative in the megakaryoblasts; the blasts may show reactivity with PAS and for acid phosphatase and punctate or focal nonspecific esterase reactivity. The megakaryoblasts express one or more of the platelet glycoproteins such as CD41, and/or CD61. In some cases, because of marked fibrosis resulting in a "dry tap" the percent of blasts is estimated from the biopsy.

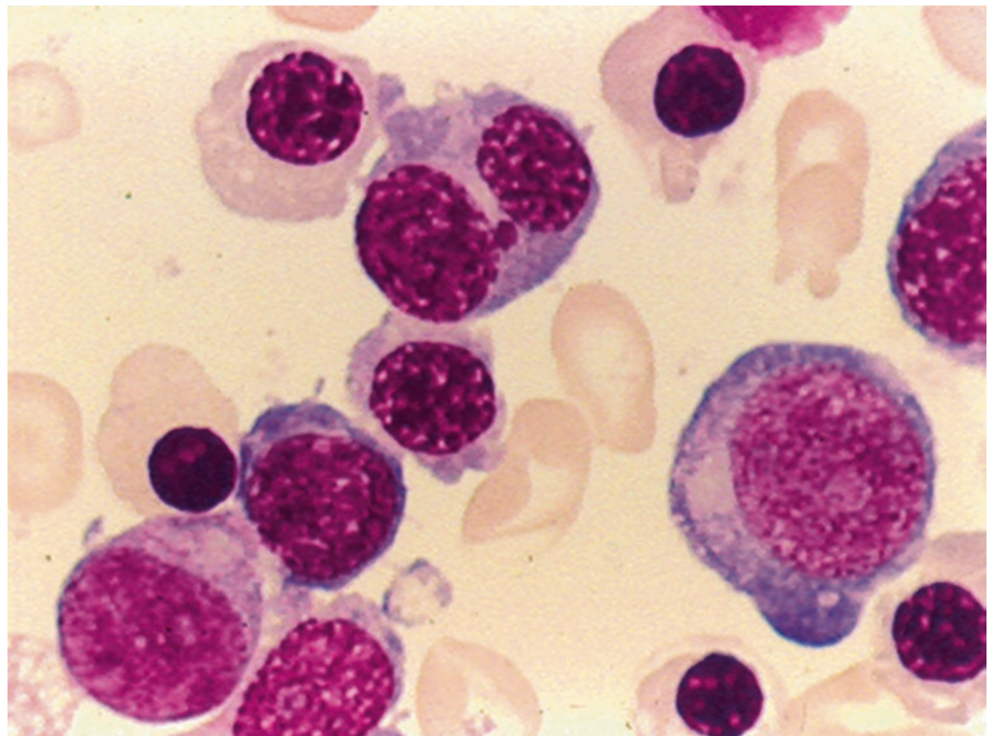

Fig. 14.20 Basophilic erythroid precursors and maturing erythroid cells. Megaloblastic changes. One myeloblast with an Auer rod (previously classified as the erythroid/myeloid subtype of erythroleukemia) WG stain

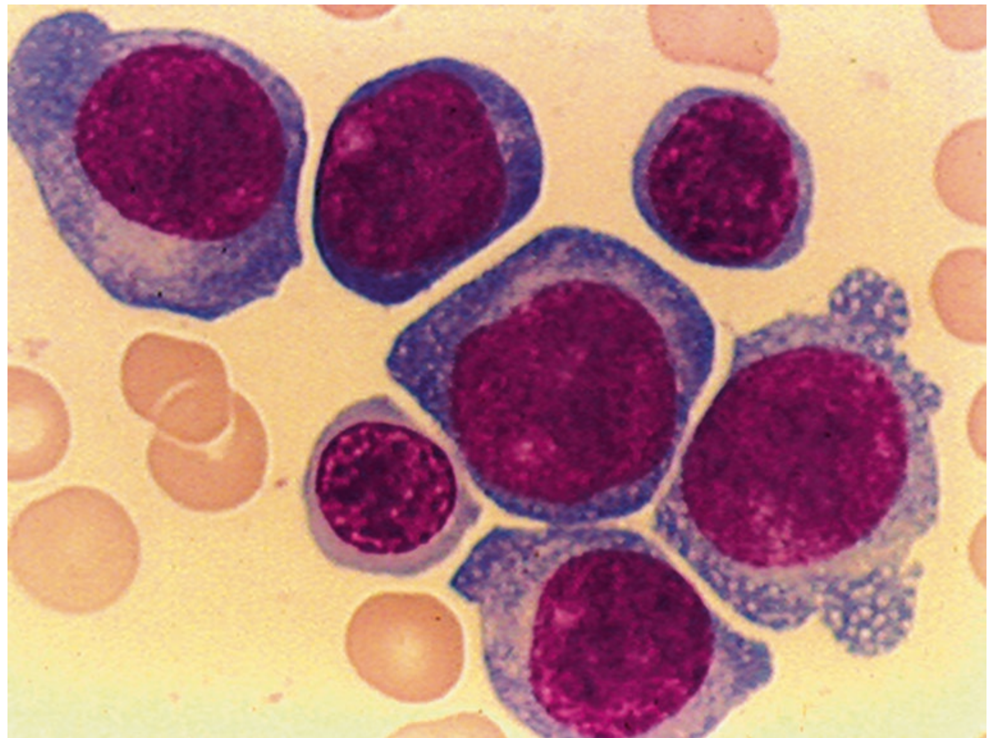

Fig. 14.21 Acute erythroleukemia (FAB M6B). No myeloblasts and many basophilic megaloblasts. WG stain

Acute Basophilic Leukemia

This is a very rare disease comprising <1% of all cases of AML. The circulating PB and BM blasts are of medium size with a high nuclear-cytoplasmic ratio; an oval, round, or bilobed nucleus characterized by dispersed chromatin; and one to three prominent nucleoli. The cytoplasm is moderately basophilic and contains a variable number of coarse basophilic granules which are positive with metachromatic stains such as toluidine blue. The differential diagnosis includes blast phase of CML, other AML subtypes with basophilia such as acute myeloid leukemia with t(6;9), AML with BCR-ABL1, and mast cell leukemia [163, 164].

Acute Panmyelosis with Myelofibrosis

Acute panmyelosis with myelofibrosis (APMF) is a very rare form of de novo AML. APMF is characterized by an acute panmyeloid proliferation with increased blasts and accompanying bone marrow fibrosis that does not meet the criteria for AML with myelodysplasia-related changes [165, 166].

The term APMF is intended to denote involvement of all three hematopoietic cell lineages. Similar features are seen in cases of MDS associated with an excess blasts and fibrosis; AML with myelodysplasia-related changes with multilineage dysplasia and fibrosis; and acute megakaryoblastic leukemia with myelofibrosis and in fibrotic phases of myeloproliferative neoplasms. The distinction between these entities and APMF may be difficult, particularly if no specimen suitable for cytogenetic analysis can be obtained.. Bone marrow biopsy supplemented with immunohistochemistry is required for diagnosis [4, 167]. Immunohistochemistry used by myeloid (MPO, CD13), megakaryocytic (CD61, CD41, factor VIII), and erythroid (glycophorin) markers confirms the presence of panmyelosis. Blasts usually express the progenitor (CD34) or more myeloid-associated (CD13, CD117) markers [4, 167, 168]. The distinction between APMF and MDS with excess blasts (MDS-EB) and myelofibrosis is very difficult since the latter cases can share most of the morphological findings seen in APMF. Cases of MDS-EB and fibrosis, except for their usually less acute clinical presentation, may be otherwise indistinguishable from APMF [4].

Myeloid Sarcoma

Myeloid sarcomas are solid myelogeneous tumors that can occur in 3–7% of AML [169], most frequently observed in children. These tumors either appear green or may become green in diluted acid because of their high MPC content (chloroma). Myeloid sarcomas can be localized in a number of locations including the skin, breast, gastrointestinal tract, lymph nodes, soft tissues, ovaries, and brain. However, most of these tumors arise adjacent to bony or neural structures [170, 171]. Myeloid sarcomas can be recognized in three types of clinical setting [6]: (1) de novo presentation without generalized bone marrow involvement (these cases should be

considered as synonymous with AML); (2) in an established diagnosis of AML either at presentation or as the first manifestation of relapse; and (3) may represent acute blastic transformation of myelodysplastic syndromes (MDS), myelodysplastic/myeloproliferative neoplasm (MDS/MPN), or myeloproliferative neoplasms (MPN) [170, 171].Myeloid sarcomas display myeloblastic, myelomonocytic, or pure monoblastic morphology [170]. The morphologic features are highly variable, ranging from little to no differentiation, where the differential diagnosis includes large-cell lymphomas and blastic plasmacytoid dendritic cell neoplasm. The histological diagnosis should be validated by immunohistochemistry (MPO, CD117, CD68, CD34, TdT, CD45, CD56 CD123, BDCA/2) [75, 172]. A more traditional stain is the specific granulocyte esterase reaction, naphthol ASD chloroacetate esterase stain on air-dried touch preparations, or non-Zenker fixed solutions. Moreover, correlation with cytogenetic and molecular genetic status is necessary to provide the correct diagnosis. AML with t(8;21), inv(16), t(1;22), and some 11q23 translocations are frequently associated with extramedullary presentations [5]. About 16% of cases carry evidence of *NPM1* mutations as shown by aberrant cytoplasmic NPM expression [173]. Inv(16) or amplification of *CBFB* has been related to breast, uterus, or intestinal involvement and possible foci of plasmacytoid dendritic cell differentiation [174].

Myeloid Proliferations Related to Down Syndrome

Patients with Down syndrome (DS) have an increased risk of leukemia. There is an approximately 50-fold increase of AML in children with DS younger than 5 years, and 70% of these neoplasms are AMKL. In contrast, AMKL comprises only 3–6% of AML in children without DS. The acute myeloblastic leukemia which occurs in children with DS has unique morphologic, immunophenotypic clinical, and molecular features [175–177] that distinguish it from other forms of AML. It commonly occurs in the first 3 years of life. Morphology of the leukemic blasts shows particular features with round nuclei and moderate amount of basophilic cytoplasm with or without blebs. Some blast contains MPO-negative coarse granules. Bone marrow core biopsy may show dense reticulin network. In these cases, antibodies to CD41 and CD61 may be particularly useful in identifying cells of megakaryocytic lineage in immunohistologic preparations. Leukemic blasts in acute megakaryocytic leukemia of DS display a similar immunophenotype to blasts in transient abnormal myelopoiesis (TAM) [178] (*positive for CD117, CD13, CD33, CD7, CD4, CD42, TPO-R, IL-3R, CD36, CD41, CD61, CD71, and are negative for myeloperoxidase, CD15, CD14, and glycophorin A*). On the other hand, TAM is a unique disorder of DS newborns in which morphologic and clinical features are indistinguishable from AML with DS [177]. The process in the majority of patients undergoes spontaneous remission within the first 3 months of life.

In addition to trisomy 21, acquired GATA-1 mutations are considered pathognomonic of TAM or AML of DS and are associated with a better response to chemotherapy and favorable prognosis compared to children with AML without DS [175]. While gene array studies have suggested differences in expression between AML of DS and TAM, however, these findings have not yet been confirmed [179].

Blastic Plasmacytoid Dendritic Cell Neoplasm

Blastic plasmacytoid dendritic cell neoplasm (BPDCN) is a clinically aggressive neoplasm derived from the precursors of plasmacytoid dendritic cells that is characterized by solitary or multiple skin lesions, often associated with regional lymphadenopathy [180]. Many cases tend to involve bone marrow and peripheral blood as well. Morphology is characterized by a diffuse monomorphous infiltrate of medium-sized blasts with scant cytoplasm, irregular nuclei, fine chromatin, and several small nucleoli. The blasts express CD4, CD56, CD43, as well as plasmacytoid dendritic cell-associated antigens (CD123, BDCA-2/CD303, TCL-1, and CLA). CD68 is expressed in 50% and TdT in 30% of cases [180–182]. In about 8% of cases CD4 or CD56 can be negative, which does not rule out the diagnosis if other PDC-associated antigens (especially CD123, TCL1, or CD303) are expressed [183, 184]. Other hematologic neoplasms especially AML with monocytic differentiation may share morphological and immunophenotypical features with BPDCN [185, 186]; an extensive immunohistochemical and/or genetic analysis is mandatory before a definitive diagnosis of BPDCN is made. On the other hand, BPDCN must be distinguished from mature plasmacytoid dendritic cell proliferation (MPDCP) in which plasmacytoid dendritic cells are morphologically mature and CD56 negative. MPDCP is invariably associated with a myeloid disorder most commonly with chronic myelomonocytic leukemia [186, 187].

Two-thirds of patients with BPDCN have an abnormal karyotype; specific chromosomal aberrations are lacking, but complex karyotypes are common [188]. Genomic abnormalities mainly involve tumor-suppressor genes [189].

The clinical course is aggressive, with a median survival ranging from 10.0 to 19.8 months, irrespective of the initial pattern of disease. Most cases (80−90%) show an initial response to multiagent chemotherapy, but relapses with subsequent resistance to drugs are regularly observed [190, 191].

Acute Leukemias of Ambiguous Lineage

Acute leukemias of ambiguous lineage show no clear evidence of differentiation along a single lineage. Cases with no lineage-specific antigens are designated as acute undifferentiated leukemia (AUL). They often express CD34, HLA-DR, and/or CD38 and TdT but lack specific myeloid and/or lymphoid antigens [192]. Leukemias that coexpress antigens of more than one lineage on the same cells or that have separate populations of blasts that are of different lineages are referred to as mixed-phenotype acute leukemia (MPAL). Some examples of MPAL blasts reveal B lineage and myeloid differentiation (MPAL, B/Myeloid) whereas other cases have both T lineage and myeloid markers (MPAL, T/Myeloid) [193, 194]. On the basis of associated cytogenetic anomalies, MPAL can be subdivided into three subgroups according to the presence of chromosome abnormalities: t(9;22)/BCR-ABL1, t(v;11q23)/MLL, or not otherwise specified (NOS) [195]. Therefore, mixed blast phase of CML must be excluded. All other unusual immunophenotypes, including early natural killer (NK) leukemias, constitute the remainder of leukemias of ambiguous origin. There may be cases that cannot be adequately classified due to insufficient immunophenotyping data or discordant expression of various markers, rendering definitive classification impossible. These cases should be designated as acute unclassifiable leukemia, which is different from AUL [196].

Central Nervous System Leukemia

Without CNS prophylaxis against CNS leukemia in ALL, isolated CNS relapse occurs in 50% of patients and is associated with subsequent systemic relapse. Therefore, CNS prophylaxis is routinely used in ALL. Although testicular and other extramedullary relapses have become exceedingly rare in ALL, in contemporary clinical trials [197] central nervous system (CNS) relapse remains a major obstacle to cure, accounting for 30–40% of initial relapses [198]. The incidence of CNS leukemia is lower in AML, and CNS prophylaxis is not usually used [199]. It should be remembered that CNS leukemia occurs frequently in patients with a prominent monocytic component (i.e., acute monocytic leukemia or acute myelomonocytic leukemia), acute promyelocytic leukemia (APL) in systemic relapse, AML with inversion or deletion of chromosome 16 (16) or chromosome 11 abnormality, hyperleukocytosis, or an elevated lactate dehydrogenase [200, 201].

The most likely explanation of CNS leukemia is direct extension from bone marrow in the skull, extending along the dura mater. Additional venules provide a direct route for leukemic infiltration of brain parenchyma [202].

Diagnostic lumbar puncture is recommended for all patients with ALL and for patients with AML who have WBC counts higher than 40,000 mm^3 [203]. Careful cytologic investigation of CNS fluid is essential to establish a diagnosis of involvement.

A CSF leukocyte count greater than 5 leukocytes/mL with the presence of unequivocal blasts on cytocentrifuge preparation is accepted as minimum cytologic criteria for CNS leukemia [204]. The two most common techniques include a cytocentrifuge preparation and a Wright-Giemsa stain or Millipore filtration technique and the Papanicolaou method. It is important to distinguish atypical but reactive cells secondary to drug-related arachnoiditis from leukemia blasts.

Evaluation of Remission

In a patient treated with acute leukemia, CR is considered when peripheral blood cell counts approach the normal range, and the bone marrow is normocellular, and shows orderly maturation of hematopoietic cells with less than 5% blast cells. The criteria for morphologic remission of less than 5% bone marrow blasts are arbitrary. The leukemic blasts in AML M3 or ALL L3, or blasts containing Auer rods, can be readily identified, even when present in small numbers. In other types of leukemia, a definitive diagnosis of residual leukemia may not be possible by morphologic examination. Persistence of dysplastic hematopoiesis in a patient who might otherwise fulfill the criteria for CR results in a much higher relapse rate. It should be remembered that the bone marrow regeneration occurs more quickly in pediatric patients (3 or 4 weeks). Therefore, one can observe a modest increase in myeloblasts and promyelocytes during recovery that may persist for 1–2 weeks. This should not be over-interpreted as a regrowth of leukemic cells.

The definition of partial remission is accepted when less than 25% leukemic cells are counted in the bone marrow smears. Relapse is defined by the presence of more than 25% leukemic cells in the bone marrow aspirate and is usually associated with peripheral cytopenias and sometimes presence of blasts.

Myelodysplastic Syndrome

Myelodysplastic syndromes (MDS) are a clonal proliferation of multipotential hematopoietic stem cells. Clinically, MDS represents a condition of bone marrow failure, usually of the elderly (primary MDS), or of patients previously exposed to prior chemotherapy or radiation or both (secondary MDS). The correlation of the biology of this clonal disorder with its clinical presentation of cytopenias is varied ranging from an incidental mild anemia stable for years to a rapidly evolving leukemia [205].

MDS is a disease of elderly, with the annual incidence of 4 cases per 100,000 in the general population.

The underlying causes in the pathogenesis of MDS remain elusive. Analyses of G6PD isoenzymes, restriction-linked polymorphisms, X-linked DNA polymorphisms of the androgen receptor (HUMARA), cytogenetic abnormalities, gene mutations, and recently targeted gene sequencing and SNP array analysis can identify somatic events in the majority of MDS patients, and have shown that MDS is a clonal abnormality of the hematopoietic stem cell characterized by defective maturation and in advanced stages uncontrolled proliferation [206–209].

Chromosomal anomalies are detected in approximately 50% of patients with de novo MDS and in up to 80% of patients with MDS secondary to chemotherapy or other toxic agent [210–212]. Balanced cytogenetic abnormalities, including reciprocal translocations, inversions, and insertions, are uncommon in MDS, in which unbalanced chromosomal abnormalities reflecting a gain or loss of chromosomal material are more prevalent [210–212]. Upcoming WHO 2008 revision recognized the importance of recent discoveries regarding the clinical significance of specific gene mutations in MDS. However, most mutations do not appear to correlate with specific disease entities with the exception for SF3B1 mutations [213, 214].

The examination of an appropriately prepared and stained bone marrow and peripheral blood smear remains the most important diagnostic approach in morphologic diagnosis [215–217]. Well-prepared thin smears with an excellent Romanowsky stains should be utilized. Iron stains are essential to address the percentage of ring sideroblasts. For an accurate differential count, at least 500 nucleated cells should be counted.

Reticulin stains prepared from bone marrow biopsy cores are recommended, since some cases will demonstrate an increased score that is viewed as impacting adversely on prognosis.

Finally, cytogenetics are critical to obtain because prognostic information is provided and is an important component of several scoring systems. Conventional cytogenetics obtained from bone marrow aspirate could be complemented by interphase FISH (with probes, i.e., 5q–,7–,8+,20q–) tests.

Morphologic Characteristics

The following discussion highlights the morphologic features used to define MDS [218–222]. In general, these features should be present in at least 10% or greater of cells of the respective lineage under consideration. The upcoming proposal is to retain the 10% threshold but provide more detailed morphologic definitions of dysplasia [217, 222–224].

Dyserythropoiesis

Morphologic bone marrow dyserythropoiesis (DysE) may include the presence of ringed sideroblasts, multinuclear fragments, bizarre nuclear shapes, internuclear bridging, mitosis, abnormal intensity of the chromatin or fine chromatin with asynchronous cytoplasm, and abnormal cytoplasmic features (intense basophilia, Howell-Jolly bodies, and ghosted cytoplasm). Marked macrocytosis (at least 100 fmol/L), basophilic stippling, anisocytosis, and poikilocytosis may be observed in the peripheral blood. Quantitative changes include the presence of ringed sideroblasts (exceeding 15% of all nucleated erythroid cells) and a number of megaloblastic erythroid precursors. However, the evaluation of dysplastic features in erythroid lineage such as megaloblastoid and cytoplasmic changes is poorly reproducible [222].

The MDS working group [51] defines three types of sideroblast: type 1 sideroblasts: fewer than five siderotic granules in the cytoplasm; type 2 sideroblasts: five or more siderotic granules but not in preinuclear distribution; and type 3 or ring sideroblasts: five or more granules in a perinuclear position, surrounding the nucleus or encompassing at least one-third of the nuclear circumference (Fig. 14.22). This definition of ring sideroblasts has been incorporated into WHO 2008 and revised WHO classification. For the definition of MDS-RS, the required number of ring sideroblasts remains at 15%.

Dysgranulopoiesis

In dysgranulopoiesis (DysG), the peripheral blood can be notable for hypogranulation and hyposegmentation of the polymorphonuclear leukocytes (PMLs) with excessive chromatin condensation (pseudo-Pelger-Hüet anomaly) (Fig. 14.23). Hypogranulation is most commonly noted and can be associated with a negative MPO reaction. The cells can be devoid of all granules, often in the more immature forms. Nuclear sticks can be seen, particularly in cases of secondary MDS or therapy-related MDS. These morphologic features may explain in part the frequency of infection in these patients (i.e., phagocytic adhesion, chemotaxis, and microbiocidal capacities may be impaired), but no correlation has been found between the loss of granules and impaired function.

Dysmegakaryopoiesis

Qualitative changes are more common in dysmegakaryopoiesis (DysM) as the number of megakaryocytes is usually normal, although hypoplasia or hyperplasia can

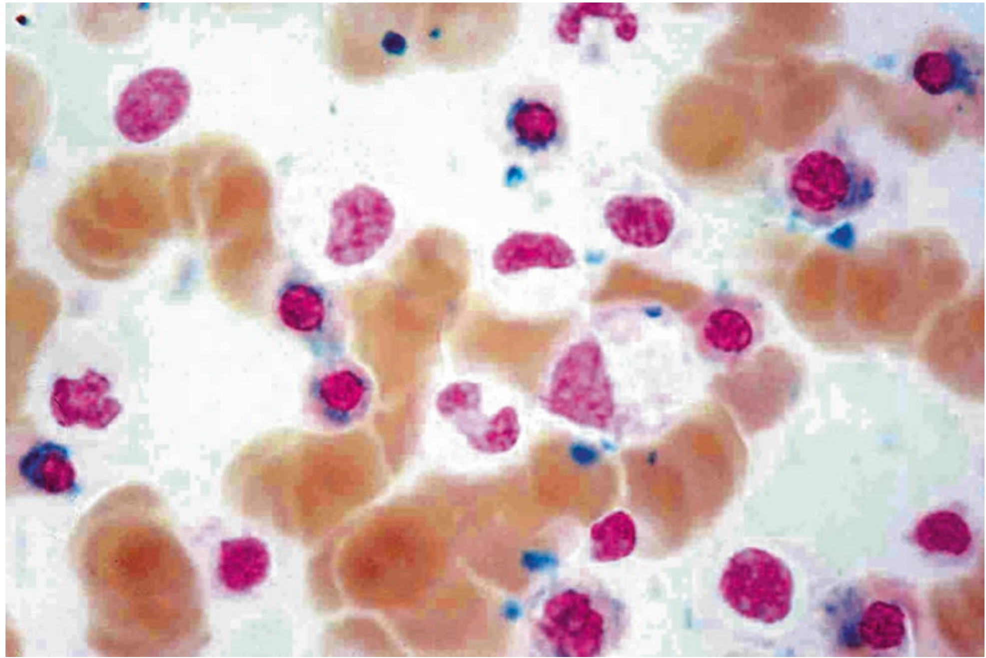

Fig. 14.22 Myelodysplastic syndrome (MDS). RARS with ring sideroblasts. Prussian *blue* reaction

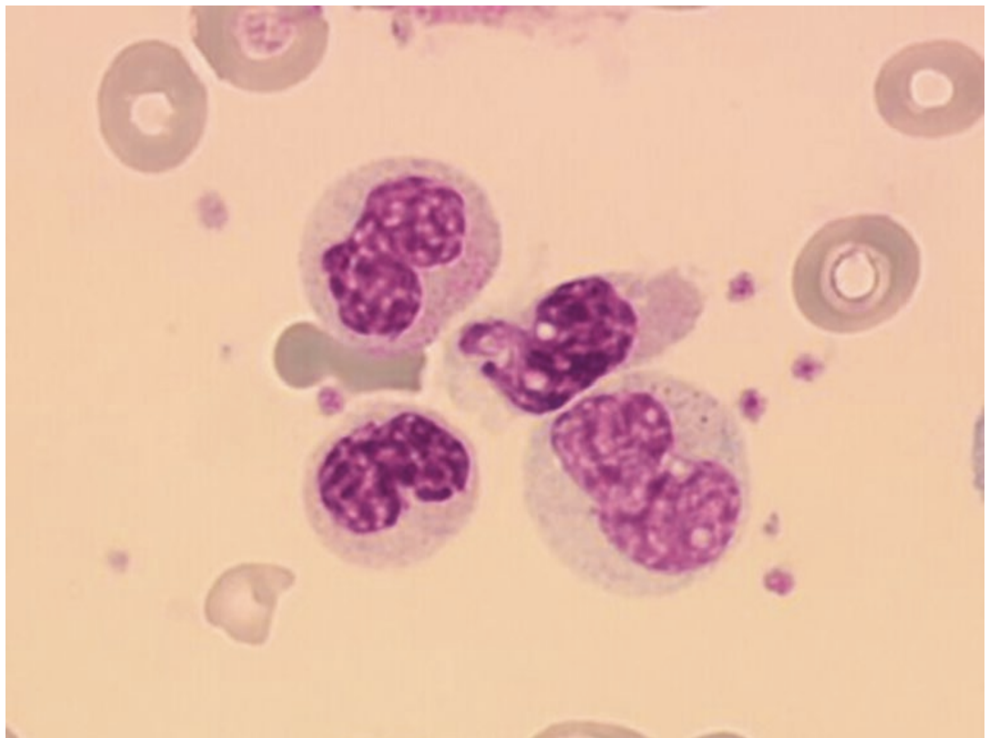

Fig. 14.23 MDS. Dysplastic granulocytes with clumped chromatin and hypogranular cytoplasm. WG stain

occasionally be seen. In the peripheral blood, large hypo- or hypergranular platelets can be found. In the bone marrow, morphologic abnormalities of the megakaryocytic precursors can be seen in half of the patients. Commonly there are micromegakaryocytes (dwarf forms) (Fig. 14.24), megakaryocytes with multiple small round, and separate nuclei and large mononuclear forms. Micromegakaryocytes can be further recognized by using CD41 and antifactor VIII antibodies on bone marrow smears with the APAAP technique. Small mononuclear megakaryocytes with a single eccentric nucleus are strongly correlated with 5q– syndrome [225].

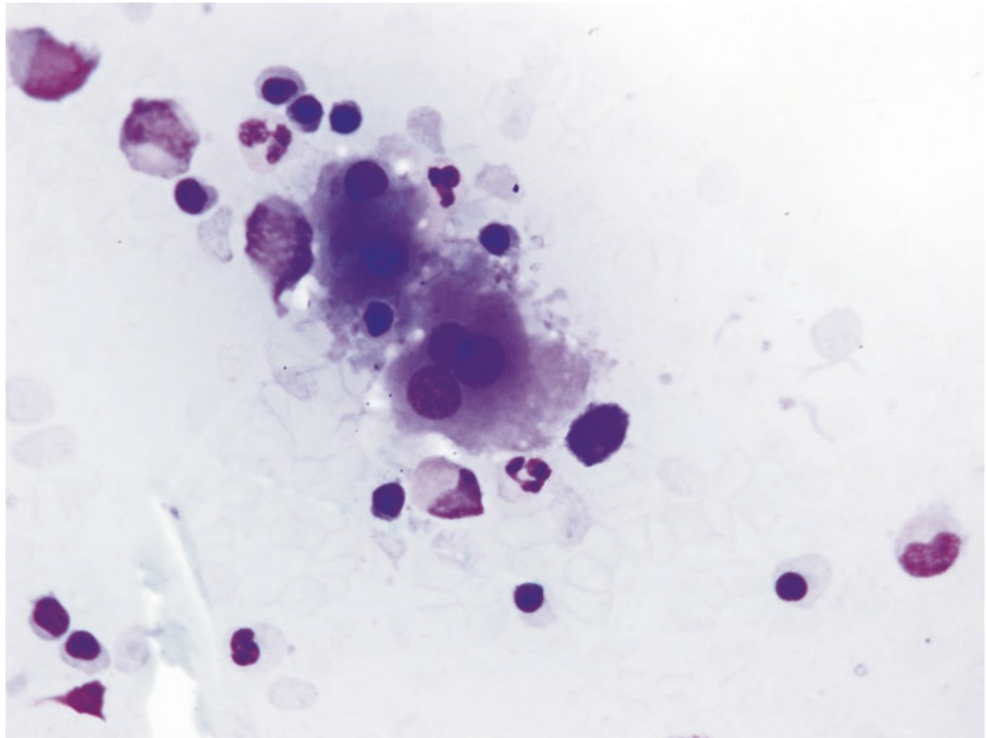

Fig. 14.24 MDS. Dysplastic megakaryocytes with separated nuclei. WG stain

Dysmonocytopoiesis

Identification of the monocyte precursors in the bone marrow is sometimes difficult, and the use of double-esterase staining may be necessary. CD14 and CD68 antibodies can also be used to identify monocytic population.

Blast Cell Characteristics

One of the most important criteria for MDS subclassification is the number (quantity) of blast cells. An area of confusion has been the identification of blasts in MDS. Recently, the international working group of morphology (IWGM) [51] recommended that myeloblasts in MDS should be classified as agranular or granular. The agranular blasts correspond to the type I blasts of the FAB classification. Granular blasts are cells that have not only the nuclear features of blast cells but also cytoplasmic granules. These cells will thus include type II blasts as defined by FAB as well as type III blasts as defined by Goasguen and coworkers [49]. Granular blasts must be distinguished from promyelocytes.

Bone Marrow Histology

The value of bone marrow biopsy in MDS is well established [64]. Bone marrow histology provides useful information on cellularity, relative proportions of three hematopoietic cell lines, architectural disorganization, and increase in the reticulin fibers.

The bone marrow cellularity should be determined as percentage of bone marrow section area according to the standard proposed by our group [226, 227]. We recommended that the bone marrow cellularity should be determined as “normocellular,” “hypercellular,” or “hypocellular” based on an age-adapted estimate.

Reticulin stain provides additional information about the degree of reticulin fibrosis which can add prognostic information.

Aggressive types of MDS (MD-EB) characterized by clusters of blast in bone marrow biopsies localized in the central part of the bone marrow away from the vascular structures and endosteal surfaces of the trabecule (Figs. 14.25 and 14.26). The blast can also be identified by immunohistochemistry using anti-CD34 antibody. Detection of blasts by anti-CD34 is especially useful in cases with fibrosis and hypocellular MDS to assess blast percentage. Previous studies have reported on the diagnostic and prognostic value of an atypical localization of immature progenitor cells (ALIP) in MDS [228]. These studies have been confirmed using anti-CD34 antibodies. In contrast to other bone marrow cells, it is possible to assess cytologic atypia of megakaryocytes in an adequately processed bone marrow sections. Megakaryocyte markers such as CD41 and CD61 are also helpful for the detection of atypical grouping or clustering and morphologic atypia of megakaryocytes. Tryptase immunohistochemistry is useful to detect coexisting occult mastocytosis [229].

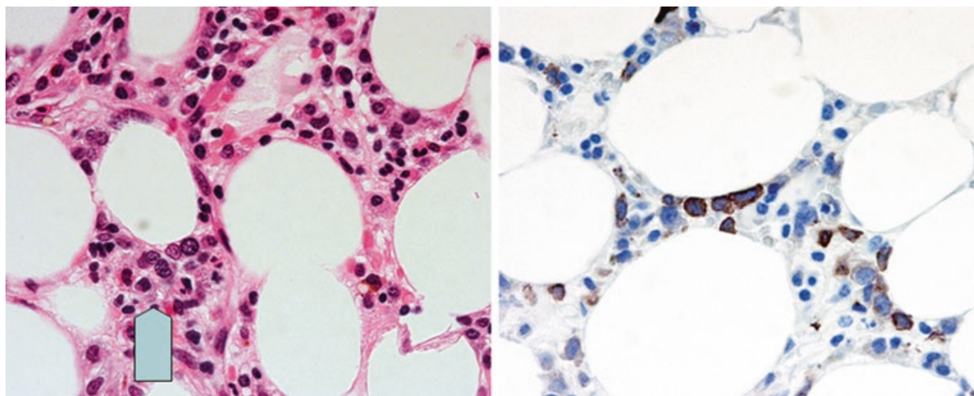

Fig. 14.25 MDS. Abnormal localization of immature precursors (ALIP). *Arrow points* to cluster and right panel shows CD34+ blasts. H&E stain

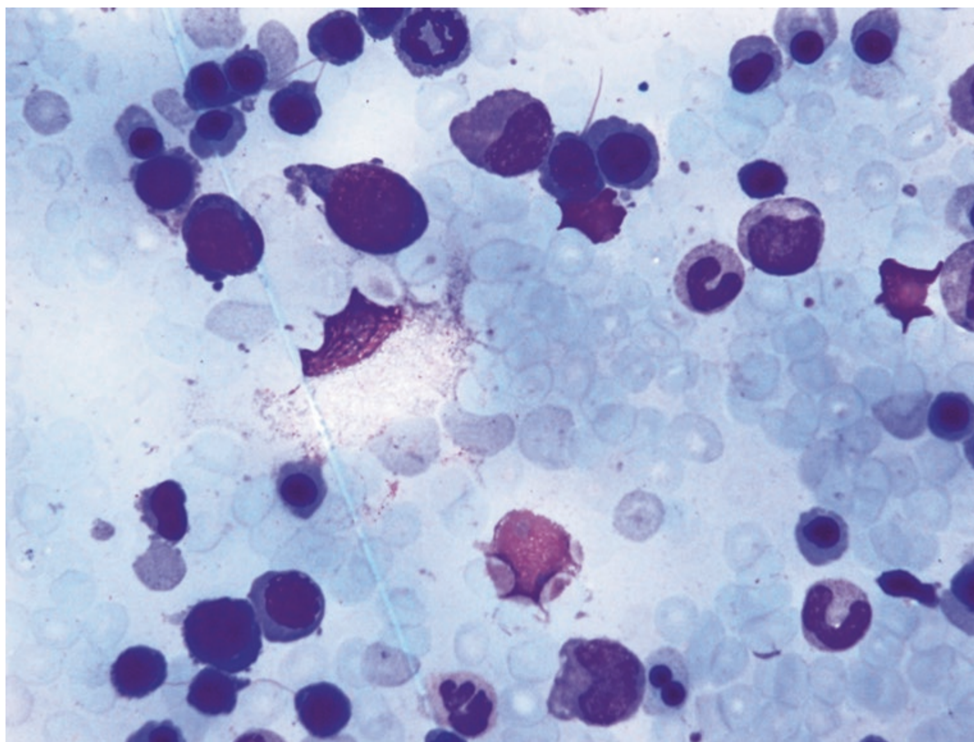

Fig. 14.26 MD-EB2. Note: increased number of blasts and megaloblastic erythroid precursors. MGG stain

Classification of MDS

In 1982, the FAB group introduced a classification system for patients with MDS [230]. The main discriminators were peripheral and medullary blast count, the percentage of ring sideroblasts, and the absolute monocyte count. This classification provided standard diagnostic procedures and became a gold standard for more than two decades.

The World Health Organization (WHO) revised and updated the MDS classification in 1997 and a revised version of the WHO classification is published in 2008 [231]. However, after 7 years, it needs to be updated. The summary of the proposed major changes to the classification related to the myelodysplastic syndromes in revised upcoming 5th edition can be summarized as follows [232]:

1- Significant changes to the morphologic criteria are not proposed. Although the current threshold of 10% to define a lineage as dysplastic may result in overcalling of dysplasia in non-MDS cases, the current proposal is to retain the 10% threshold but provide more detailed morphologic definitions of dysplasia and emphasize the importance of carefully considering non-MDS causes of dysplasia [217–224].
2. Blast cell counts resulted in no change so the classification will continue to recommend that blast cell counts should ideally be performed on well-stained, cellular bone marrow aspirate smears; although the utility of CD34 staining on a trephine biopsy may be helpful for blast cell estimates in the absence of cellular aspirate smears, such as in the setting of marrow fibrosis.

3. The use of cytopenias versus morphologic dysplasia in defining MDS subtypes is sometimes leading to confusion. It is proposed to replace the terminology of "refractory anemia" and "refractory cytopenia" with "myelodysplastic syndrome" in the revised classification [i.e., MDS with single-lineage dysplasia (MDS-SLD)].
4. The cytogenetic criteria for MDS will likely remain unchanged.
5. In WHO 2008 classification RCMD-RS was incorporated in RCMD; however this category is reinstated as RCMD-RS in upcoming edition. MDS cases with SF3B1 mutations have a distinctive gene expression pattern [233], with a large number of differentially expressed genes [234]. This combination of shared morphology (ring sideroblasts) and a shared underlying driver mutation (SF3B1) now favors separating MDS with ring sideroblasts as distinct entities, which may have single or multilineage dysplasias. Thus, MDS cases with SF3B1 mutation can be classified as RARS or RCMD-RS if any ring sideroblasts are present. Ring sideroblasts and SF3B1 mutations also occur in high-grade MDS with excess blasts and even in AML. It should be remembered that RARS or RCMD-RS only defines a specific MDS entity in cases with <5% bone marrow blasts.
6. Recent studies have shown that the del(5q) abnormality in MDS is prognostically similar whether it is isolated or occurs with one additional low-risk cytogenetic aberration [235]. Based on this finding, the category of MDS with isolated del(5q) will be expanded to encompass cases with one additional non-high-risk cytogenetic abnormality. Blasts are <5% in bone marrow, and <1% in blood; dysplasia is uni- or multilineage but cases with significant myeloid dysplasia are excluded [236].
7. The category of MDS unclassified in the revised 2008 classification is retained and includes patients with pancytopenia and unilineage dysplasia, and patients with no overt dysplasia but cytogenetic evidence of cytogenetics. Unilineage dysplasia cases with 1% blood blasts are detected on at least two separate occasions.
8. In upcoming revision erythroid/myeloid type of erythroleukemia with >50% erythroid precursors and 5–19% blasts are now considered as MDS-EB rather than AML. Acute erythroid/myeloid leukemia is linked with MDS, since they share both morphologic and genetic features [237, 238]. Cases with ≥20% blasts and >50% erythroid precursors will still be classified as AML with myelodysplastic changes. Pure erythroleukemia will remain in AML.
9. A provisional entity, refractory cytopenia of childhood (RCC), has been added in 2008 classification to include children with cytopenia(s) with less than 2% peripheral blood and less than 5% bone marrow blasts and evidence of dysplasia in two or more lineages. It remains as a provisional entity in the updated classification.

Morphologic Subtypes

MDS with Single-Lineage Dysplasia (MDS-SLD)

MDS with single-lineage dysplasia (MDS-SLD) comprises 10–20% of all cases. It is primarily disease of elderly. The vast majority of MDS-SLD cases are associated with unilineage erythroid dysplasia (RA). Anemia is the main manifestation (hemoglobin below 11 g/dL with a low reticulocyte count) with variable dyserythropoiesis. Blast cells usually are not present in the peripheral blood (<1%) and fewer than 5% in the bone marrow. Dyserythropoiesis varies from slight to moderate. The bone marrow biopsy is generally hypercellular but may be normocellular or even hypocellular. In general, RA can be considered as a "low-grade" MDS with median survival in the range of 6–7 years and only 5–10% of cases progressing to overt acute leukemia [239, 240]. Other forms of MDS-SLD are rare.

MDS with Single-Lineage Dysplasia and Ring Sideroblasts (MDS-RSSLD)

The morphologic features of MDS with ring sideroblast single-lineage dysplasia (MDS-RSSLD) are similar to those of MDS with single-lineage dysplasia (MDS-SLD) except that there are more than 15% ringed sideroblasts in the bone marrow. However, patients with ring sideroblasts but not meeting the 15% threshold used to define MDS-RSSLD will still be diagnosed if an SF3B1 mutation is detected. Macrocytosis and dysmorphic red cells are present in the peripheral blood (Fig. 14.27). MDS-RSSLD accounts for 3–11% of all MDS cases. It is primarily disease of elderly with a median age of 60–73 years [232, 239]. Ring sideroblasts represent erythroid precursors with abnormal accumulation of iron within mitochondria [51]. It should be remembered that ring sideroblasts are frequently observed in other types of MDS (i.e., MD-EB). If the platelet count is 450 × 103/μL (450 × 109/L) or greater and the megakaryocytes have features of those described in the myeloproliferative neoplasms (MPNs), an analysis for JAK2 and SF3B1 mutations is indicated. Most of these cases may be assigned to the provisional entity of RA with ring sideroblasts and thrombocytosis [241] which is considered within the MDS/MPN group.

MDS with Multilineage Dysplasia (MDS-MLD)

MDS-MLD accounts for approximately 30% of all MDS cases. It is a disease of elderly. The median age is 70 years [232]. Bone marrow aspiration and/or biopsy are usually hypercellular and characterized by erythroid, granulocytic, and megakaryocytic dysplasias. Bone marrow blasts are less than 5%. Auer rods are not seen. If present those cases should be classified as MDS-EB2. The clinical course varies. Patients with RCMD have a worse outcome (reported median survival of 17–33 months) than patients with RA [240]. The frequency of AML evaluation at 2 years is approximately 10%.

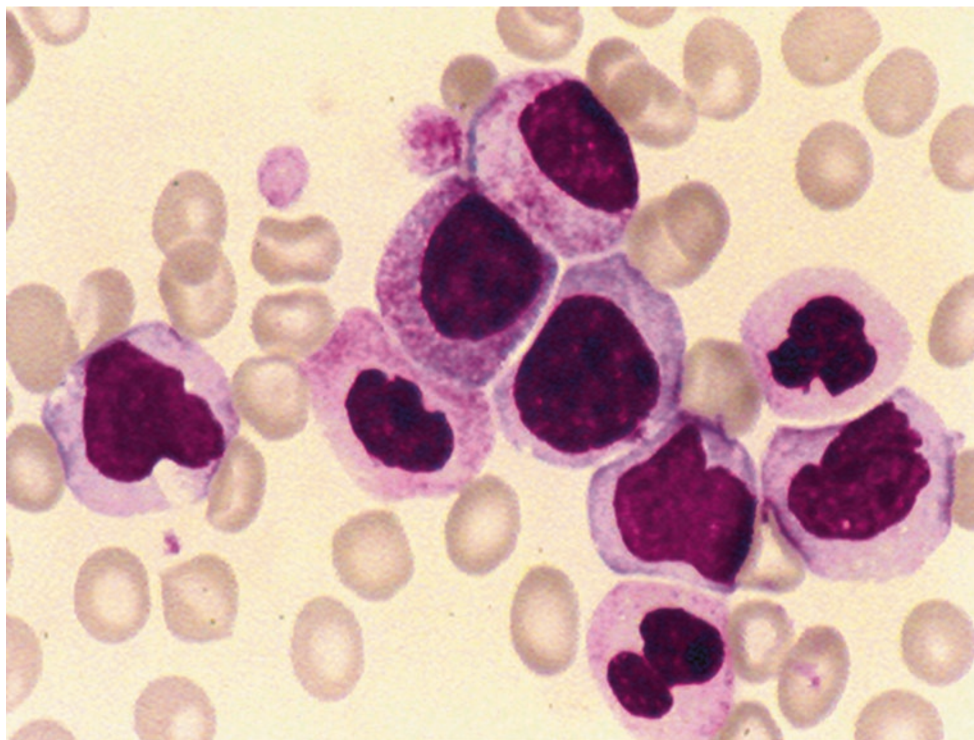

Fig. 14.27 MDS. CMML. Monocytic precursors and myeloblasts. WG stain

MDS with Multilineage Dysplasia and Ring Sideroblasts (MDS-RSMLD)

The category of RCMD-RS was originally eliminated in the 2008 WHO Classification and merged with RCMD because it was shown to be prognostically similar to RCMD lacking ring sideroblasts [242]. Although this still appears to be the case, the recent discovery of mutations in the spliceosome gene SF3B1 that are associated with ring sideroblasts provided a link between morphology and genetics in MDS [243]. Other morphologic features, except ringed sideroblasts, are similar as MDS with multilineage dysplasia.

MDS with Excess of Blasts (MDS-EB)

Conspicuous changes in all three lineages are present associated with variable cytopenia affecting two or more of the hematopoietic lines. Blasts (granular and agranular) range from greater than 5% but less than 20% in the bone marrow or 2–19% in peripheral blood. Two categories of MDS with excess of blasts (MDS-EB) are recognized: MDS-EB1, defined by 5–9% blasts in the bone marrow or 2–4% blasts in peripheral blood, and MDS-EB2, defined by 10–19% blasts in the bone marrow or 5–19% blasts in the peripheral blood [240]. The presence of Auer rods in blasts qualifies a case as MDS-EB2 regardless of the blast percentage. The bone marrow biopsy is very useful in documenting the presence of blast clusters particularly in cases with extensive fibrosis or hypocellularity. Blasts in MDS-EB tend to form cell aggregates that are usually located away from the trabecule and vascular structures. CD34 staining is helpful in their identification. Patients with MDS-EB2 have worse survival and a higher rate of disease transformation to AML. Median survival time for MDS-EB1 is approximately 18 months vs. 10 months for MDS-EB2 [240].

MDS with Fibrosis

Moderate-to-severe (Grades 2–3) bone marrow fibrosis is observed in 10–15% of patients with MDS. These cases have been referred to as MDS with fibrosis [244]. Most of these cases belong to the MDS-EB category. These cases may morphologically overlap acute panmyelosis with fibrosis (APMF). It has been shown that bone marrow fibrosis represents an independent prognostic parameter and identifies a distinct group of MDS with multilineage dysplasia, high transfusion requirement, poor-risk cytogenetics, and poor prognosis. Furthermore, the presence of CD34+ cell clusters is an independent risk factor for progression to AML [245].

Myelodysplastic Syndrome with Isolated del (5q)

Myelodysplastic syndrome with isolated del (5q) is characterized by severe anemia, absence of or mild neutropenia, and/or thrombocytosis. Blast cells usually are not present in the peripheral blood (<1%) and fewer than 5% in the bone marrow. There is marked female predominance. Bone marrow hypercellular or normocellular frequently exhibits erythroid hypoplasia. Megakaryocytes increase in number. Small mononuclear hypolobated

megakaryocytes with single eccentric nucleus and many granulations are strongly correlated with isolated del(5)q [225]. Recent studies have shown that the del(5q) abnormality in MDS is prognostically similar whether it is isolated or occurs with one additional low-risk cytogenetic aberration [246]. Based on this finding, the category of MDS with isolated del(5q) will be expanded to encompass cases with one additional cytogenetic abnormality (excluding monosomy 7).

MDS in Children

Primary MDS is very rare in pediatric population and accounts to less than 5% of all malignant hematopoietic neoplasms among children under the age of 14 years [247]. Although many morphologic and genetic features are common in adult and pediatric cases, there are some significant differences present, particularly in patients with low-grade MDS (RA, RARS, del.5q) categories. Unlike adults, children with MDS present with thrombocytopenia in approximately 75% of cases [248]. Moreover, hypocellularity of the bone marrow is more commonly observed in children than in adults [249]. For these reasons, a provisional entity, refractory cytopenia of childhood (RCC), has been added to include children with cytopenia(s) with less than 2% blasts in the peripheral blood and less than 5% in the bone marrow and evidence of dysplasia in two or more lineages. It remains as a provisional entity in the updated classification for children with 2–19% blasts in the blood and/or 5–19% in the bone marrow; the MDS subclassification should be made using the same criteria used for adults.

Hypocellular Myeloid Neoplasms

Hypocellular AML and hypocellular MDS represent small (10–15%) but significant number of patients diagnosed with myeloid malignancies [250, 251]. Hypocellular AML affects elderly and accounts for 5–12% of de novo AML. In a recent study although the outcome of hypocellular acute myeloid leukemia does not differ from that of non-hypocellular acute myeloid leukemia, hypocellular AML is characterized by prominent cytopenias, older age, a high percentage of antecedent hematologic disorders or prior chemotherapy/radiotherapy, and a low frequency of proliferative mutations [252]. Hypocellular MDS is more frequent in women and occurs with an age-related frequency which is similar to that seen in primary MDS. Bone marrow cellularity is the critical determinant to recognize hypocellular myeloid neoplasm and a bone marrow biopsy is necessary to diagnose these variants in all patients including children. According to our experience [228], by using anatomic comparisons, 13% of AML and 29% of MDS patients had hypocellular marrows. Correcting for age lowered the percentage of hypocellular marrows 2.2% and 7%, respectively. Therefore, age correction is necessary and should be considered as one of the defining criteria for such diagnoses. Bone marrow cellularity may be an important prognostic factor in hypocellular MDS. There is some controversy on survival differences between hypocellular MDS and normo/hypercellular MDS, but patients appear to do at least as well or better [251, 253, 254]. The separation between hypocellular AML, hypocellular MDS, and aplastic anemia (AA) can be problematic. Most hypocellular MDS cases fall into the categories of MD-SLD and MD-EB. The presence of easily identifiable megakaryocytes and patchy erythropoiesis with defective maturation within an architecturally disorganized marrow and the presence of reticulin fibrosis favor MDS over aplastic anemia [249, 250, 253, 255]. An important feature provided by the bone marrow biopsy is to identify blasts. In patients with myeloid neoplasia, blasts often form clusters in central marrow cavity location in contrast to paratrabecular location in reactive marrows. The presence of such clusters is mainly seen in the aggressive MDS subtypes. Immunohistochemical stains, mainly CD34, have provided additional assistance for counting blast in tissue sections. Increase in the percentage of CD34/CD117-positive blast cells and a tendency to form aggregates are useful in distinguishing hypoplastic myeloid neoplasms from aplastic anemia [256]. Although cytogenetic studies may be of particular value in this group of disorders, cytogenetic failures are frequently observed due to severe hypocellularity. FISH studies on paraffin sections or from peripheral blood may be useful for the detection of particular chromosome abnormalities (i.e., 7–, 5–). A recent study revealed that single-nucleotide polymorphisms-array (SNP-A) karyotyping in aplastic anemia and hypocellular MDS can complement metaphase cytogenetics and lead to the identification of cryptic clonal genomic aberrations in both disorders leading to improved distinction of these disease entities [257].

Myelodysplastic/Myeloproliferative Diseases

The WHO classification [9] recommends that a separate category to be formed to include disorders that have both myelodysplastic and myeloproliferative features including CMML, JMML, and aCML. These disorders have many common features including abnormalities of both granulocytic and monocytic lines and a relatively aggressive course.

Chronic Myelomonocytic Leukemia

The criterion for the diagnosis of CMML is the presence of peripheral absolute monocytosis higher than 1×10^9/L, associated with a marrow proliferation of monocytes. For most cases, the peripheral blood and bone marrow smears of patients fulfill all of the classical criteria for MDS (variable degrees of trilineage dysplasia) and show identical chromosome abnormalities. In the WHO classification [258], CMML is further divided into two subcategories, depending on the number of blasts and promonocytes found in the bone marrow and peripheral blood: CMML1, blasts and promonocytes

less than 5% in the peripheral blood and less than 10% in the bone marrow, and CMML2, blasts and promonocytes 5–19% in peripheral blood and 10–19% in the bone marrow. Presence of Auer rods qualify as CMML2 regardless of the blast percentage. The value of this approach has been validated [259].

A subset of patients with eosinophilia, which are formerly included in the CMML category associated with genetic abnormalities including PDGFR, are classified as myeloid neoplasms with eosinophilia.

The percentage of bone marrow and peripheral blasts and the presence of cytogenetic aberrations have been associated with shorter survival and a higher risk of AML evolution [260]. Moreover, the presence of EZH2 implies an unfavorable prognosis [261] while mutation of ASXL1 correlates with evolution to AML and a shorter overall survival [262]. The median survival time varies from 20 to 40 months in most reported series. Progression to AML occurs in 15–30% of cases.

Atypical Chronic Myeloid Leukemia, BCR-ABL1 Negative

This disease predominantly involves the neutrophilic series as CML but lacked Ph1 chromosome or BCR/ABL translocation. Therefore, it is renamed as atypical CML. BCR/ABL negative emphasizes importance of obtaining these tests. aCML has dysplastic and proliferative features. Dysgranulopoiesis is a constant finding. Leukocyte alkaline phosphatase score (LAP) is not useful for diagnosis. Specific and nonspecific esterase stains are useful to detect monocytic component and exclude CMML. aCML is a disease of elderly but has also been reported in young patients. Its prognosis is significantly worse than Ph1+ CML and other MDS/MPNs with a median survival of 14–30 months, and an acute myeloid leukemia (AML) progression rate of approximately 40% [263]. MPN-related mutations are either absent or very infrequent in aCML, and the detection of CSF3R T618I, MPL, CALR, or JAK2V617F mutations should prompt a differential diagnosis of chronic neutrophilic leukemia (CNL), primary myelofibrosis (PMF), or myeloproliferative neoplasm-unclassifiable (MPN-U), which can share overlapping features with aCML [264].

Juvenile Myelomonocytic Leukemia

This is a separate disorder seen in children and adolescents and distinct from adult CMML. Blasts and promonocytes account for less than 20% of cells in both bone marrow and peripheral blood. Erythroid and megakaryocytic dysplasias are frequently seen [265]. Young patient age (median 1.8 years), predominant hepatosplenomegaly, frequent skin involvement, leukocytosis, monocytosis, and presence of immature precursors in peripheral blood characterize juvenile myelomonocytic leukemia (JMML). Ten percent of patients are known to have neurofibromatosis type I by clinical criteria [266]. It is a rapidly fatal disorder; however it rarely transforms into AML. Approximately 90% of patients carry either somatic or germline mutations of PTPN-11, K-RAS, N-RAS, CBL, *or* NF1 in their leukemic cells. These genetic aberrations are largely mutually exclusive and activate the Ras/mitogen-activated protein kinase pathway [267, 268].

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Immunobiology of Acute Leukemia

15

Elisabeth Paietta

Introduction

Acute leukemias are commonly defined as the expansion of immature cells that are derived from rare transformed hematopoietic progenitor cells, termed the leukemic stem cells (LSC) or leukemia-initiating cells (LIC), which have the capacity of self-renewal, a defining characteristic also of normal hematopoietic stem cells [1]. As an alternative to originating in a primitive stem cell, leukemias may be derived from transformation of a committed progenitor cell, as is the case with the PML/RAR**α** fusion gene which confers self-renewal to the promyelocyte compartment [2]. The specific cell surface phenotype of LSCs is CD34$^+$, CD38$^-$ [3]. To distinguish between normal and malignant CD34$^+$, CD38$^-$ cells, additional markers are required, such as the C-type lectin-like molecule (CLL-1) [4], CD123 [5], and CD47 [6]. Remarkably, however, even this basic LSC phenotype may vary among patients, such as in truly CD34$^-$, often *NPM1* (nucleophosmin 1)-mutated AML [7], or in leukemias with rearrangement of the *MLL* (mixed-lineage leukemia) gene, in which surface antigen expression of LSCs depends on gene fusion partners [8]. Together with the loss of long-term repopulating potential, the phenotypic features of the LSC blast cell progeny that invade the bone marrow and the peripheral blood of leukemia patients are different from those of the LSC and typically characterized by different morphology and antigen profiles. Despite limited morphologic variability, which forms the basis of the FAB classification, the genetic heterogeneity of both acute myeloid (AML) and lymphoid leukemias (ALL) is vast. With very few exceptions, namely hypergranular *PML/RARα^{POS}* acute promyelocytic leukemia (APL) associated with FAB M3, the t(8;21) and inv(16) core-binding factor (CBF) leukemias associated with M2 and M4Eo, respectively [9], and *RUNX1* mutations, which are associated with the M0 FAB type [10], the morphologic appearance of leukemic cells lacks predictive power with respect to their genetic makeup. While FAB classes may still be collected as part of the biologic characterization of study cohorts, they are no longer part of multivariate analyses establishing the prognostic significance of mutational landscapes [9–15].

For some of the cytogenetic/genetic aberrations, specific antigen profiles have been established, termed surrogate marker profiles, which, in itself, are independent of the underlying genetic lesion, e.g., for APL, for *AML1/ETO* (now termed *RUNX1/RUNX1T1*), and for *BCR/ABL^{POS}* ALL [16, 17]. For other genotypic subtypes, such as BCR/ABL1-like or Ph-like ALL, overexpression of the cytokine receptor-like factor 2 (CRLF2), found in approximately 50% of cases [18], can be reliably detected with an antibody to surface expression of CRLF2 by the leukemic B-lymphoblasts when compared to CRLF2 analysis by fluorescence in situ hybridization [19]. The 2016 revision to the World Health Organization (WHO) classification of myeloid neoplasms and acute leukemia [13] illustrates clearly the increased impact of genetic aberrations in the standardized classification of the acute leukemias. The newly defined concept of pre-leukemic cell clones which carry recurrent mutations, particularly of epigenetic regulator genes (*DNMT3A, TET2*), has revealed novel insights into the intricacies of hematopoiesis and the development of acute leukemia [20–22]. These pre-leukemic stem cells are resistant to standard chemotherapy, persist during remission, and may correspond to a patient's risk of relapse. The fact that these somatic mutations are found with astonishing high frequency in clonally expanded hematopoetic stem cells of healthy subjects [23–26], however, may challenge the notion that pre-leukemic stem cells are the most important target for minimal residual disease (MRD) monitoring.

Antigens expressed by leukemic cells are identical to those expressed by normal hematopoietic cells. To date,

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there is no evidence for the existence of leukemia-specific antigens, with the exception of proteins that are encoded by novel fusion genes as a result of leukemia-specific chromosomal aberrations [27] or by mutated genes, e.g., *NPM1* [28]. The value of these antibodies to leukemia-specific proteins lies in their ability to detect genetic aberrations by alternative techniques.

The concept of surrogate marker profiles for genetic lesions [16, 17] follows the basic hypothesis that molecular features determine the cellular phenotype. For leukemic clones, this notion implies that the expression of (altered) genes associated with leukemogenesis will cause characteristic distortions of the cellular phenotype with some predictable consistency that can be exploited by sophisticated immunophenotyping. This explains the occurrence of unique antigens or antigen patterns in leukemia subtypes characterized by particular cytogenetic and/or molecular abnormalities. Examples are the typical antigen profile of AML with t(8;21) *(RUNX1/RUNX1T1*), the classical marker composition of *PML/RAR*α^{POS} APL, and the association of CD25, the α-chain of the interleukin-2 receptor, with BCR/ABL^{POS} ALL, all of which will be discussed in detail.

Unfortunately, reliable surrogate marker profiles for genetic lesions are rare and even the finding of a CD34^{-}, HLA-DR^{-}, CD11a^{-}, CD18^{-} phenotype, which is strongly indicative of APL [29], is only that, indicative of APL, and must be confirmed by the molecular presence of the *PML/RAR*α-fusion gene by the polymerase chain reaction (PCR) assay. On the other hand, elevated surface expression of CRLF2 by B-lymphoblasts is almost invariably associated with the Ph-like CRLF2 phenotype [18, 30]. It does not, however, distinguish between *IGH@-CRLF2*, *P2RY8-CRLF2*, and other kinase fusions.

The characterization of universal markers for poor treatment response with current treatment strategies is another aspect of antigen expression worth mentioning. Besides CRLF2, CD25 is one such antigen both in B-lineage ALL [31] and AML [32]. Other antigens exhibit their unfavorable prognostic significance only in association with a certain maturation stage of acute leukemia, such as the poor outcome associated with expression of the myeloid antigen CD13 in cortical, mature but not in immature T-ALL [33].

The finding that particular combinations of antigens expressed by leukemic cells may be rarely or never be seen in normal hematopoietic tissues has important implications for the detection of MRD, whether the different-from-normal approach or the concept of leukemia-associated immunophenotypes (LAIP) is used [34]. The best example is the differentiation between hematogones, normal B-cell precursor cells, and leukemic B-lymphoblasts while monitoring MRD in B-lineage ALL, which requires extensive knowledge of the intricate antigen expression by hematogones and their various differentiation stages [35, 36]. With more sophisticated immunophenotypic analyses, it has become apparent that leukemic cells differ from normal hematopoietic cells and that leukemia categorization cannot be based solely on presumed normal counterparts [37]. Of course, it is still valid to group the acute leukemias according to the major hematopoietic cell lineages (T-lymphoid, B-lymphoid, myeloid), based on the expression of lineage-specific antigens, and to distinguish between precursor and more mature subtypes based on the hierarchy of antigen expression observed in normal hematopoiesis. However, refined, clinically relevant subgrouping focuses increasingly on prognostically significant and/or therapy-determining features, which may cross lineage borders, as seen, for instance, with the CD25 antigen, mentioned above. The 2008 WHO classification of neoplastic diseases of the hematopoietic and lymphoid tissues [9] and its recent revision [13] clearly reflect this tendency by increasingly recognizing cytogenetic/molecular categories in the new nomenclature of the acute leukemias, thereby reducing those disease entities which rely on morphology and/or immunophenotype alone and invariably suffer from reduced prognostic significance. Comprehensive genomic profiling can now be performed in the clinical setting to improve the diagnosis of hematologic malignancies [38, 39] and more precisely match patients with targeted therapies [40]. The emphasis on marker compositions and single antigens which are associated with genetic features and/or carry prognostic significance irrespective of lineage affiliation should be viewed as a new role for immunophenotyping that deviates from mere lineage identification.

Clinical flow cytometrists today most commonly are still charged solely with determining the lineage affiliation of a given leukemia population to provide an immunodiagnosis which is then routinely compared with the morphologic evaluation of the same tissue under the microscope and, hopefully, with cytogenetic data, to provide the diagnostic basis for therapy. There remains the challenge of accurately interpreting the rare true bilineal (BLL) or mixed-phenotype acute leukemia (MPAL), though any controversies as to whether T/MPALs or B/MPALs [9, 13] should be treated as lymphoid or myeloid leukemias are determined by their genetic makeup rather than their presumed lineage affiliation. I am saying presumed lineage affiliation, because recognizing true lineage affiliation depends on the detection of lineage-specific, predominantly intracellular antigens, which remain to present a challenge to many. Genomic characterization of immunophenotypically distinct subpopulations from two patients with T/myeloid BLL failed to demonstrate genetic diversity, suggesting that the cells with T-, myeloid-, or T/myeloid features all derived from a common LSC with multilineage differentiation capacity [41]. This concept of inherent leukemic plasticity [37, 42, 43] emphasizes that treating BLL or MPAL leukemias according to the dominant phenotype seen at the time of testing is ill advised; genotypic features should guide the treatment decision.

Inevitably, routine clinical flow cytometry laboratories are limited in their efforts due to costs and time restraints. Even if involved in clinical trials with protocol-derived correlative studies, the laboratory must strike a balance between routine immunophenotyping for diagnostic purposes and science-driven, prognostically pertinent testing, if only limited material is available and more sophisticated correlatives, such as genomic studies, must be performed on the same tissue. Ideally, the number of cells required for mere diagnosis-confirming flow cytometry is reduced to a minimum by using multiple fluorochromes in combination and a selection of most informative antibodies. This chapter discusses the various roles of immunophenotyping and the relation between antigen expression and other biologic or clinical aspects in the acute leukemias. In particular, it emphasizes on novel developments, such as the exciting developments in immunotherapy, MRD detection, and novel genotype-driven leukemia subsets. The discussion of lineage-affiliated antigen expression and immunophenotypic classification systems, areas which have been introduced extensively in the previous editions [44, 45], this time will be touched on only peripherally, as far as they relate to the other topics.

Immunophenotyping Versus Morphology

The classification of the acute leukemias has relied on the evaluation of cell size, granularity, nuclear shape, cytoplasmic appearance, cytochemical reactions, and dysplastic features of cells surrounding the "leukemic blasts" since the introduction of the French-American-British (FAB) classification system first in 1976, followed by more recent modifications [46]. Twenty years later, when the WHO published its first proposed classification for hematopoietic tumors [47], FAB terms for ALL were no longer found relevant. For AML, the new classification encompassed four major categories: (1) AML with recurrent genetic abnormalities; (2) AML with multilineage dysplasia; (3) AML and myelodysplastic syndromes, therapy related; and (4) AML not otherwise categorized. In the 2008 WHO edition [9], category 1 was significantly expanded by incorporating more balanced chromosomal translocations and inversions as well as a list of gene mutations that had been found to be of prognostic significance and/or associated with a normal karyotype in the years between the two publications. Despite these additions, which to some extent reduced the number of cases previously left uncategorized, particularly in elderly AML [48], a large group of patients still remained in category 4, not otherwise categorized molecularly and therefore still classified by FAB criteria. Proteomic profiling confirmed the ability of the FAB classification to distinguish between the major lineages by aligning protein signatures with myeloid (M0, M1, M2), monocytic (M4, M5), or M6/M7, though without discriminating the degree of maturation [49]. This may explain why FAB classes are usually not good predictors of prognosis, with the exception of FAB M3 or hypergranular APL. Critics of the WHO classification blame it for emphasizing simplicity of classification at the expense of prognostic significance [50].

An excellent example for underappreciated discordance between information provided by FAB criteria and the actual level of maturation arrest indicated by antigen expression in a recurrent cytogenetic aberration is t(8;21) or *RUNX1/RUNX1T1*$^{\mathrm{POS}}$ AML. Incredible effort has gone into accurately identifying this AML subtype based on morphology. Nucifora et al. [51] proposed a scoring system for the identification of t(8;21) AML based on a list of morphologic characteristics—a total score of 6 or 7 would be suggestive of t(8;21). This scoring system was subsequently adjusted to capture more t(8;21) positive cases while simultaneously decreasing the false-positive rate from 12% with the Nucifera score to 7% with the weighted score [52]. This better recognition rate was accomplished simply by altering the scores assigned to the various morphologic features, reflecting the randomness of such scoring systems. Although predominantly associated with FAB M2 (AML with maturation) [9], 20% of t(8;21) AML cases presented with M1 features (AML without maturation) upon central morphology review (Bennett JM, personal communication) of a recently completed phase III trial of the Eastern Cooperative Oncology Group (ECOG) in >600 younger AML patients [53]. A characteristic feature of the t(8;21) AML immunophenotype is a striking myeloid immaturity, such as weak or absent expression of CD33 [52] and/or the more mature myeloid antigen, $\mathrm{CD65}_{(\mathrm{S})}$ (Paietta E, personal observation), and failure to detect myeloperoxidase by antibody binding [54], and consistent with the suggestion that the *RUNX1/RUNX1T1* fusion event occurs at an early stem/(progenitor cell stage [55]). This lack of myeloid maturation has mostly gone unnoticed due to the continuing tendency of pathologists to place more weight on morphology rather than immunophenotype, particularly in AML. The unique B-lymphoid antigenic features of t(8;21) AML, with expression of PAX5, CD19, and CD79a [56], are a further indication for focusing on refined immunophenotyping, especially since immunostaining for PAX5 is a valuable diagnostic tool for t(8;21) AML presenting with extramedullary masses, which occur frequently in this disease [9]. The absence of expression of the T-lymphoid antigen, CD7, in t(8;21) AML was reported many years ago [52, 57]. More recently, however, we described a subtype of AML which resembles closely the phenotype of t(8;21) AML but expresses CD7 together with CD19 [58]. Strikingly, this novel AML presents with a normal karyotype, lacks *RUNX1/RUNXT1* transcripts, but instead contains internal tandem duplications of the *FLT3* gene (*FLT3ITD*^{POS}) and mutated *NPM1* (*NPM1MUT*^{POS}).

An intriguing observation is the application of immunophenotyping in the discovery of the genetic diversity of morphologically defined APL. Flow cytometry recognizes the characteristic cellular structures of hypergranular (FAB M3) APL by producing a scattergram that reflects cells of large size (high forward-angle light scatter, FSC) with a high degree of granularity (high 90° angle scatter or side scatter, SSC). However, for variant hypogranular APL cells (FAB M3v), the scatter signal can be quite variable and misleading [59]. In a report from the Europena Working Party [60], the majority of cases with FAB M3 or M3v morphology contained t(15;17)(q22;q12)/PML/RARα or other cytogenetic aberrations involving the RARα gene. To account for a small subgroup of morphologic APL patients apparently lacking a RARα rearrangement, a new morphological subclass was introduced and termed "M3r," which also covered APL presenting with PLZF/RARα transcripts. Although the immunophenotype of M3r cases shared the negativity for CD34 and HLA-DR, which is part of the surrogate marker profile for t(15;17) APL [29], M3r patients lacked response to all-trans retinoic acid (ATRA) in vivo. In other words, the main rationale for classifying APL, namely to recognize ATRA-responsive disease, was invalid in this morphologic subtype. The other lesson that should have been learned from these data was that the standard CD34- and HLA-DR-negative marker profile, still widely accepted as sufficient for the diagnosis of APL, is unreliable in predicting the disease. Contrary to this overzealous attempt to define APL by morphology, t(15;17) leukemia without M3/M3v features is often ignored. Among larger studies [59, 61], the incidence of such APL cases ranges between 1 and 2%. For these as well as patients with cryptic t(15;17) [59], the improved surrogate antigen profile for PML/RARα^{POS} APL applies, consisting of negativity for HLA-DR, CD11a, and CD18 [29]. While this antigen combination pertains to both M3 and M3v morphologies, there are immunophenotypic peculiarities that are limited to M3v and/or leukemic promyelocytes containing the S-isoform of PML/RARα transcripts; these are expression of CD34 and of the T-cell-affiliated antigen, CD2 [29]. In fact, CD2^{POS} leukemic promyelocytes frequently lack all of the antigenic properties associated with APL. In such cases, expression of CD2 by myeloid blasts per se is the strongest indication that one is dealing with an APL. It is important to remember that CD2 expression in AML is rare and if present most commonly suggests APL or AML FAB M4Eo with inv(16)(p13q22) resulting in the *CBF*β*/MYH11* fusion transcript [62]. In confirmation of a biologic heterogeneity within the APL phenotype, results from gene expression profiling demonstrated that M3 and M3v APL were clearly separable [63]. Furthermore, the origin of CD2^{POS} APL has been localized to a progenitor cell more immature than that of CD2^{NEG} APL [64].

The role of morphology in the diagnosis of "acute leukaemias of ambiguous lineage" [9] is difficult to assess, since this leukemia subtype is frequently erroneously diagnosed. Occasionally, two morphologically distinct blast populations are readily distinguishable in individual patients; still, even in such cases, the demonstration of lineage-specific antigens is essential for the differential diagnosis of "mixed" (distinct expression of lineage-specific antigens in the two morphologically different populations) versus "biphenotypic" leukemia (expression of lineage-specific antigens by one and the same blast cell irrespective of morphology). More common is the finding of antigens specific for more than one cell lineage in a morphologically homogenous blast cell population, e.g., myeloperoxidase and cytoplasmic CD22 (cCD22) or myeloperoxidase and cytoplasmic CD3 (cCD3), the antigens specific for the myeloid, B-lymphoid, and T-lymphoid lineage, respectively. An instructive example for such a true biphenotype is that of CD117^{POS}, FLT3-gene mutated T-lineage ALL [65]. This rare leukemia subtype simultaneously expresses cCD3 and, in a subset of blast cells (5–10%), myeloperoxidase. Despite the presence of the myeloid-specific antigen in only a minor portion of the blasts, the morphology of these cases varied between FAB M2 (occasionally with Auer rods) and FAB L2, independent of the percentage of myeloperoxidase^{pos} blasts.

Minimally differentiated AML is considered to be equivalent to FAB M0, with no evidence of myeloid differentiation by morphology or cytochemistry [9]. Based on a retrospective analysis of >700 adult AML patients across all ages, ECOG defined this undifferentiated phenotype based on lack of expression of the more mature myeloid antigen, CD65(s), a carbohydrate antigen that can be expressed in an asialostate (CD65) or with a sialic acid residue in terminal position of the carbohydrate chain (CD65s) [66]. Morphologically, the majority of these patients belonged to the FAB M0/M1 classes. Important hints in the correct interpretation of this AML subtype are the following: (a) blasts must be negative for lymphoid-specific antigens, cCD22 and cCD3; (b) although blasts do not show an enzymatic reaction for myeloperoxidase, they frequently stain for the myeloperoxidase protein by antibody staining; (c) even if blasts are negative with antimyeloperoxidase antibody, they are considered myeloid given that they lack lymphoid-specific antigens; (d) in most cases, the blasts will stain for CD33 and/or CD13, the pan-myeloid antigens; (e) immature antigens, such as CD34, CD117, HLA-DR, CD133, and CD123, are common; (f) frequently, the T-lymphoid-affiliated antigen CD7 is found; (g) expression of the B-lymphoid antigen CD19 by these undifferentiated blasts suggests t(8;21)/AML1/ETO^{POS}AML. Minimally differentiated AML more often occurs in older patients and carries inferior prognosis [66].

In summary, diagnoses based on morphology cannot be easily correlated with immunophenotypic findings. With the exception of FAB-M3, morphologic subclasses rarely relate to specific immunophenotypes. The presence of monocytes and/or monocytic features in a leukemia population, however, requires special discussion. Invariably, when CD14, the prototype mature monocytic antigen, is detected on an abnormally

large fraction of white blood cells (WBC) or on blasts cells defined by the expression of immature markers, such as CD34 or CD117, the morphologic evaluation will yield FAB M4 (myelomonocytic) or M5 (monocytic), respectively. It should be emphasized here that CD123 (α-chain of the IL-3 receptor), an antigen associated with the hematopoietic stem cell [67] and expressed by >99% of AML [68], cannot be used to diagnose leukemic monocytes, because normal monocytes show weak, though persistent, CD123 expression. The strong association of CD14 with monocytic morphology stands in contrast to CD11b (Mac-1), an integrin α-subunit (adhesion molecule) [69], which in monocytic development appears earlier than CD14 [70] and which is also expressed by myeloid cells starting at the myelocyte stage [71]. Given its appearance late in myeloid maturation, one would expect CD11b expression by nonlymphoid leukemic cells to reflect monocytic features. However, in a retrospective evaluation of 382 patients with CD11b^{POS}CD14$^{NEG/LOW}$ AML, ECOG found that less than half of those patients belonged to FAB classes M4/M5, whereas the other half demonstrated FAB M1/M2 features [72].

An instructive tale relates to the "cuplike" nuclei which have been published as a distinct morphologic feature in AML with FLT3-gene internal tandem duplication (FLT3-ITD) [73]. According to Kussick et al. [73], non-APL AML-cuplike was more likely to lack HLA-DR and CD34, to express CD123 without CD133, and to have a normal karyotype. A few years later, Chen et al. [74] associated the same prominent nuclear invaginations with NPM1-mutated AML, which is characterized by negativity for CD34 and CD133, normal cytogenetics, and a high incidence of FLT3 gene mutations. These structural nuclear features prevail in NPM1-mutated AML irrespective of FAB categories, which typically span a wide spectrum in this genetic subtype. Furthermore, NPM1 mutations involve all but lymphoid hematopoietic cell lineages and, as FAB criteria, also this feature is independent of the presence of FLT3-ITD [75]. While previously [47] included in the class of "AML not otherwise characterized" and subjected to diagnosis based on FAB criteria, the newest WHO classification [13] recognizes NPM1-mutated AML as a distinct disease category rather than mere prognostic factor.

Immunophenotypes Versus Genetic Lesions

Chromosome translocations can have two distinct effects at the molecular level: either the inopportune activation of an unaltered gene or the creation and transcription of a novel gene. The first process occurs predominantly in lymphoid malignancies. The translocation places a transcriptionally silent gene under the control of the promoter of a transcriptionally very active gene, e.g., immunoglobulin or T-cell receptor (TCR) genes in B- or T-lymphocytes, respectively [76]. This leads to the inappropriate transcription of a normal gene. Alternatively, balanced translocations and interstitial chromosome deletions or inversions can lead to the creation of novel, leukemogenic fusion genes. At each of the chromosomal breakpoints, a critical gene is disrupted; fragments of the two genes are brought together as a result of the translocation. Two hybrid fusion genes are created, one on each of the two chromosomes partnering in the translocation. Even if both chimeric genes are transcribed, only one is usually suspected as the transforming gene.

The following section is limited to those leukemia fusion genes, gene mutations, and instances of aberrant gene overexpression for which immunophenotypic data are available. While some antigen combinations can be viewed as specific for certain genetic lesions (e.g., AML1/ETO^{POS} AML), others are indicative of a certain maturation stage of leukemic arrest (e.g., BAALC-overexpressing leukemias) or reflective of a specific cell of origin (e.g., NPM1-mutated AML).

Surrogate Antigen Profiles for Leukemia Fusion Genes

Specific associations have been established between the expression of single antigens or particular antigen expression patterns and cytogenetic-molecular abnormalities. While the first of such surrogate marker profiles were found rather serendipitously, i.e., that for t(15;17)/PML/RARα APL, antigen expression signatures of genetic lesions are increasingly sought in a planned fashion, in parallel to the genetic classification of the acute leukemias as well as due to developments in targeted therapy [16, 17]. Reliable surrogate marker profiles for genetic lesions are of clinical interest only when the associated genotype is prognostically significant and/or when targeted therapy for that genotype is available.

Outcome-based classification of the leukemias nowadays is based predominantly on cytogenetic aberrations and their molecular derivatives, whether recognizable by standard cytogenetics or requiring FISH analysis. Progressively, however, genetic and epigenetic profiling data provide a major source of information regarding perturbed pathogenetic pathways and potential therapeutic targets. The revolutionary discovery of BCR/ABL1-like or Ph-like B-lineage ALL is the best example for this development [18, 19, 30]. The advantage of having antigen profiles available, which can predict for the existence of, for example, gene mutation or gene silencing events, lies in the ease, speed, and low costs of flow cytometry. Appropriate situations of particular urgency arise when cytogenetics are unsuccessful or simply not performed, when chromosome structures are impaired, as in ALL, when only normal metaphases are seen, or when karyotyping, FISH analysis, and/or molecular analyses are not available at an institution. Surrogate marker profiles should not be used in lieu of molecular analyses, given that even the most reliable antigen combinations have shown

some unexpected properties: (a) sensitivity to subtle antigenic alterations outside the core marker profile; for example, the antigen profile for t(8;21)-*AML1/ETO*^{POS} AML is invalid when the T-cell marker CD7 is also present on the surface of the myeloblasts; (b) precise alterations in response to variations in the global genetic lesion; for example, striking differences were found in the expression of CD25 and dual CD33/CD13 between the *e1a2* and *e13a2(b2a2)/e14a2(b3a2)* transcript forms in *BCR/ABL*^{POS} ALL, and absence of CD33/CD13 expression in the presence of a del(9p) in addition to the t(9;22); and (c) occasionally an overpowering effect of single antigens; for example, the expression of the T-cell marker CD2 by leukemic promyelocytes occasionally obviates the finding of the typical surrogate marker profile for *PML/RAR*α APL.

t(15;17)(q22;q12)-*PML/RAR*α^{POS} APL

Given the unmistakably hypergranular features of M3 leukemic promyelocytes, which rarely cause misreadings among morphologists, APL was the first leukemia for which characteristic immunophenotypic features were described. The history of the surrogate marker profile for APL is outlined in Table 15.1. The initial definition of an APL marker profile consisted of negativity for CD34 and HLA-DR. Subsequently, the profile was refined by adding the differential reactivity of CD15/$CD15_S$ antibodies, caused by the sialylation of the CD15 carbohydrate antigen in APL, weak expression of CD38 and CD45, expression of CD9, and lack of P-glycoprotein [17]. Two developments prompted the search for an improved profile: (1) the recognition of an ATRA-unresponsive AML subtype, termed natural killer cell AML, with morphologic similarities to M3v as well as CD34 and HLA-DR negativity, while expressing CD56, the neural cell adhesion molecule [77], and (2) the observation that CD56 expression by leukemic promyelocytes conferred inferior prognosis [78, 79]. The most recent surrogate marker profile for PML/RARα APL [29] is based predominantly on (a) lack of HLA-DR and CD133, two antigens expressed at differentiation levels more immature than that of promyelocytes during normal myelopoiesis; (b) absence of several adhesive molecules, such as CD11a (α_L subunit of the leukocyte integrin LFA-1), CD18 (β_2 subunit of LFA-1), and CD11b (α_M subunit of Mac-1 integrin); (c) expression of the carbohydrate structure, CD15, only in the sialylated form, $CD15_s$; and (d) faint expression of CD45, the common leukocyte antigen, and of CD38, a bifunctional ectoenzyme catalyzing involved in cell adhesion to endothelium. In summary, APL is characterized by absence or weak expression of adhesion molecules. CD117, a progenitor molecule associated predominantly with the myeloid lineage, is invariably expressed by leukemic promyelocytes, albeit occasionally at low levels [29, 59].

Table 15.1 History of the immunophenotype of *PML/RAR*α^{POS} APL

Earliest definition	Current definition	ECOG's new definition
Myeloid	Myeloid	Myeloid
HLA-DR NEG	HLA-DR NEG	HLA-DRNEG
CD34 HLA	CD34 NEG	CD11a NEG
	CD15 NEG	CD18NEG
	CH15s POS	CD133 NEG
	CD9 POS	CD45 WEAK
	CD38 LOW	CD38 WEAK
	Pgp WEAK/NEG	CD15 NEG

"Myeloid" defines the presence of a basic myeloid phenotype with expression of CD33 and CD13, occasional expression of $CD65_{(S)}$ and/or myeloperoxidase, and absence of lymphoid-specific antigens. *Pgp* P-glycoprotein, the multidrug resistance protein; *NEG* (negative) reflects absence of antigen-expressing cells; *POS* (positive) reflects expression of antigens by >10% of gated leukemic promyelocytes; *WEAK* reflects weak intensity of fluorescence of antibody staining

The (15;17) translocation involves the retinoic receptor α (RARA) gene on the long arm (q) of chromosome 17 and the promyelocytic leukemia (PML) gene on the q arm of chromosome 15. While the breakpoints in the RARA gene occur consistently in intron 2, differential breakpoints in the PML gene lead to the L- (Long, bcr1), S- (Short, bcr3), or the V- (Variable, bcr2) transcript isoform of PML/RARα. The HLA-DR^{LOW}, CD11a^{LOW}, CD18^{LOW} surrogate marker profile is applicable to all three molecular isoforms [29, 59]. However, the isoforms can be distinguished based on specific antigenic features outside this core antigen profile. While the antigen expression patterns in L- and V-form diseases are indistinguishable, they are clearly separable from S-isoform APL. Only leukemic promyelocytes that contain S-form transcripts variably express CD34, CD2, and CD56. The expression of CD2 together with the S-isoform correlates with poorer prognosis [80]. A potential explanation could be a higher incidence of extramedullary relapse in CD56^{POS} cases [81]. Furthermore, thrombotic events in APL were found to be associated with CD2^{POS} S-form disease with *FLT3-ITD* [82]. As discussed before, CD2^{POS} APL is hypothesized to be derived from a progenitor cell with myeloid/lymphoid potential [63, 83]. Note that while CD2 and/or CD56 on a patient's leukemic promyelocytes are highly suggestive of the S-isoform, because they are never seen in L- or V-isoform, there are S-isoform patients who lack these antigens. CD34 expression may be associated with the molecular isoform or the microgranular morphologic variant (see before). Even when expressed, the density of the CD34 antigen is significantly lower on the surface of leukemic promyelocytes than on non-APL myeloblasts [84]. CD2^{POS} APL occasionally may lack APL-typical antigenic features, something never seen in L- or V-form APL, thus possibly misleading laboratory investigators or pathologists when they interpret the data [16, 17]. It is recommended that any case of CD2^{POS} myeloid leukemia be immediately tested by PCR for *PML/RAR*α versus *CBF*β*/MYH11*, since APL [59] and inv(16)(p13q22)/t(16;16)(p13;q22) AML [62], respectively, are the two major AML subtypes found to express CD2, especially since these leukemia subdiagnoses require distinct therapies.

In addition to the *PML/RAR*α fusion gene, which accounts for >98% of APL cases, a common segment 5′truncated *RAR*α has been found to fuse with alternative genes [85]. Because such variant translocations are rare, clinical information regarding their responsiveness to ATRA is scarce; recurrent cases of *PLZF/RAR*α (promyelocyte leukemia zinc finger), derived from t(11;17)(q23;q21), appear to lack ATRA responsiveness, while *NPM/RAR*α (nucleophosmin) APL, derived from t(5;17)(q35;q21), is ATRA responsive. Despite their low frequency, the limited immunophenotypic observations available suggest that the main characteristic features of *PML/RAR*α APL cells hold up for all currently known variant APL translocations that involve rearrangement of the *RAR*α gene [59]. Novel variant *RAR*α fusion genes keep appearing in the literature whereby ATRA sensitivity in individual cases appears to vary. Two pieces of evidence should prompt the search for the presence of an alternative *RAR*α fusion gene in a patient: (a) the finding of APL-specific immunophenotypic features in a patient negative for *PML/RAR*α, and (b) cytogenetic evidence of chromosome 17 abnormalities in such a patient. Occasionally, slight variations from the typical APL profile may be found. Gallagher et al. [86], for instance, found weak expression of CD133 in the only second case of *STATb/RAR*α. If confirmed in further cases, this antigenic peculiarity may serve as a diagnostic tool for this particular APL variant.

t(8;21)(q22;q22)-*RUNX1/RUNX1T1* (formerly *AML1/ETO*)^{POS} AML

Leukemias with t(8;21) or inv(16)/t(16;16) belong to the core-binding factor (CBF) AMLs, which are a diagnostically and prognostically distinct subgroup [9]. Both of these chromosomal rearrangements result in the formation of fusion proteins, *RUNX1/RUNX1T1* and *CBF*β*/MYH11*, respectively, that involve the disruption of one of the CBF transcription factor genes. The two genes involved in (8;21) translocation are the AML1 transcription factor, now called *RUNX1*, on chromosome 21q22.3, and the eight-twenty one oncoprotein (ETO) (now called *RUNX1T1*) on chromosome 8q22 [55]. The characteristic immunophenotype of t(8;21) AML allows for a correct prediction of this genetic aberration [87]. Distinctive features are expression of CD19, a B-lineage-associated antigen, and of CD56, the neural-cell adhesion molecule, by CD34^{POS} myeloblasts. Presence of CD56 may explain the increased incidence of granulocytic sarcomas observed in this disease [88, 89]. While CD19 is consistently present in the t(8;21) subtype and rarely found in other AMLs, expression of this antigen by myeloblasts is often very weak so that its detection can depend on the accurate choice of fluorochromes and an open mind on the part of the interpreting flow cytometrist. In fact, CD19 conjugated to fluorescein isothiocyanate (FITC) should be avoided at all costs. An example of CD19/CD56 double expression in a patient with *RUNX1/RUNX1T1*^{POS} AML is shown in Fig. 15.1. Although CD56 expression is promiscuous among myeloid leukemias and absent in a marked fraction of t(8;21) cases, the finding of both CD19 and CD56 against the background of a myeloid phenotype is highly suggestive of t(8;21) AML. As in the case of APL, an

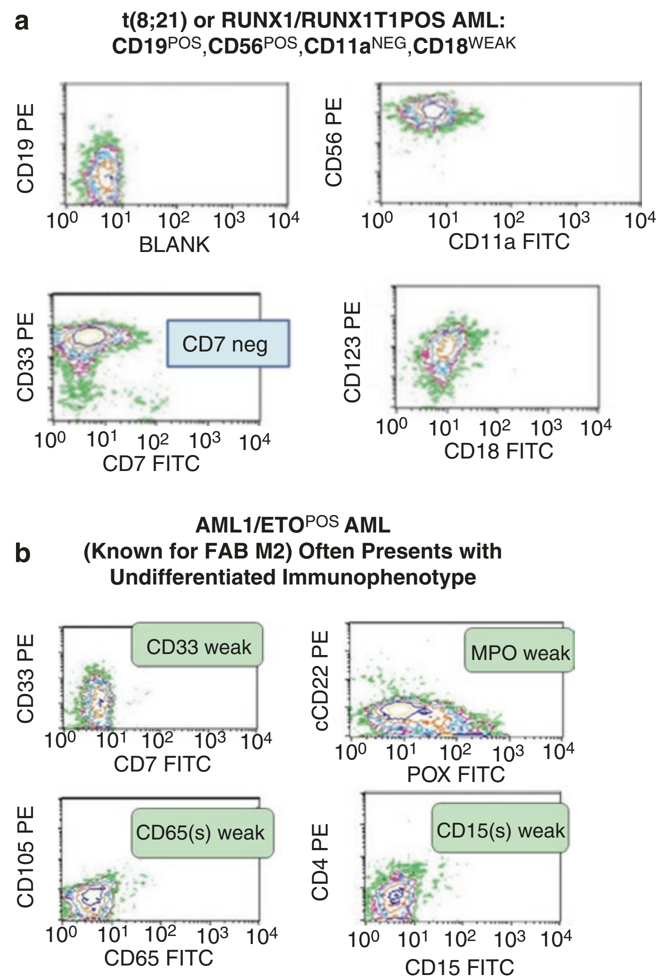

Fig. 15.1 (**a**) Dual-CD19 and -CD56 expression by t(8;21)-*RUNX1/RUNX1T1*^{POS} myeloblasts. Blasts were gated based on low side and forward scatter and all antigen expression shown reflects that of gated blasts. Antibody staining along the *X*-axis demonstrates fluorescence intensity of FITC-conjugated antibodies, whereas the *Y*-axis represents PE-conjugated antibody staining. (*A*) This contour plot demonstrates the weak though definite expression of CD19. (*B*) The same weakly CD19^{POS} blasts also strongly express CD56 but lack CD11a. (*C*) CD33 expression in this particular case of *RUNX1/RUNX1T1*^{POS} AML is strong, but notably there is no CD7 expression. (*D*) The blasts express the stem cell marker, CD123, but lack CD18. (**b**) This case of *RUNX1/RUNX1T1*^{POS} AML demonstrates the frequently found immature myeloid phenotype. Blasts were gated as described under 1.A. (*A*) Blasts only weakly express CD33 and lack CD7. (*B*) Myeloperoxidase expression is weak but there is no evidence of intracytoplasmic CD22. (*C*) Mature myeloid antigens $CD65_{(s)}$ and CD15 (*D*) are absent. The blasts also fail to stain for CD105, a marker of immature hematopoietic cells, and CD4, which is frequently expressed by monocytic cells. In the presence of CD19 staining (not shown for this case), this pattern of antigen expression may be confused with CD10^{NEG} B-lineage ALL. To exclude B-lineage ALL, myeloperoxidase and intracytoplasmic CD22 must be evaluated. Furthermore, CD10^{NEG} B-lineage ALL blasts preferentially express CD65/CD15 rather than CD33

increasingly accurate surrogate marker profile for t(8;21) AML has evolved over time. One particularly helpful diagnostic tool is the diminished or absent expression of CD11a/CD18 [59, 90], a member of the β2 integrin subfamily [69]. With the exception of t(8;21) or t(15;17) (and its variants), >90% of AML demonstrate expression of CD18. The absence of CD11a in t(8;21) AML is explained by the inhibition of Runx1-dependent CD11a transcription by the *RUNX1/RUNX1T1* fusion product [90].

Variable expression of CD11a is seen in AML with dual expression of CD19 and CD7, a T-lineage-affiliated antigen [58]. Previously, the incompatibility of t(8;21) with expression of CD7 had been reported [57]. More recently, CD19/CD7 double-positive AML has been associated with a predominantly normal karyotype and *FLT3-ITD* and *NPM1* mutations [58] (Fig. 15.2).

Aside from CD19, t(8;21) myeloblasts can also express two other B-lineage antigens, CD79a and PAX5 [56]. The *PAX5* gene encodes a paired box domain transcription factor, which is considered a crucial mediator of B-cell identity [91]. Tiacci et al. [56] hypothesized that the PAX5-dependent expression of CD19 and CD79a in t(8;21) AML results from the interaction and functional cooperation between the PAX5 and AML1/ETO proteins. Expression of CD56 is also not limited to the myeloid lineage [92] and in B-lineage ALL appears to be associated with BCR/ABL transcripts [93]. Taken together, these findings can lead to a misdiagnosis of B-lineage ALL, especially in view of the immaturity of the myeloid phenotype frequently encountered (as discussed before), as long as karyotypic and molecular data are unavailable. While in t(8;21) AML, the same blast population will co-express myeloperoxidase or surface myeloid antigens, e.g., CD13, weak CD33, and CD19, these blasts will not stain for cCD22. It is important to remember that dual absence of myeloperoxidase and cCD22 is consistent with AML.

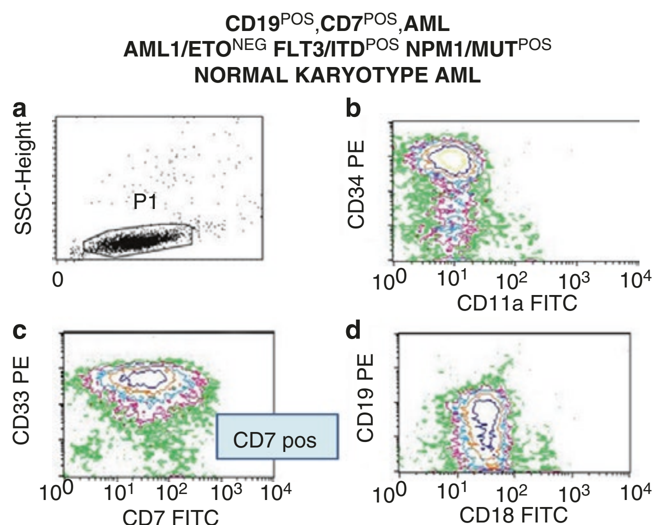

Fig. 15.2 Expression of CD7 by CD19^{POS} myeloblasts rules out t(8;21)-*RUNX1/RUNX1T1^{POS}* AML. (**a**) P1 is drawn around suspicious cells with low side (SSC) and forward scatter (FSC), reflecting lack of granularity and small-to-intermediate cell size. (**b**) In this biparametric contour plot, cells within gate P1 are found to contain a major population with high-intensity CD34 expression and a minor population with variable, weak CD34 expression; both populations lack CD11a. (**c**) Further characterization of gated cells reveals that both CD34^{HIGH} and CD34^{WEAK} cells belong to the leukemic population as they all express both CD33 and CD7. (**d**) These CD33^{POS}CD7^{POS} myeloblasts also express CD19 and lack CD18. In graphs b–d, a small population of residual normal T-lymphocytes is seen. In graph b to the right of the main CD34^{POS} cell population is a very small component of CD11a^{POS} cells; in graph c there is a small component of CD7^{POS} but CD33^{NEG} cells, and in graph d, there is a small population of CD18^{POS} but CD19^{NEG}, suggesting that they reflect a small number of normal T-lymphocytes, which had been inadvertently included in the P1 gate

t(9;22)(q34;q11)-*BCR/ABL^{pos}* ALL

The Philadelphia chromosome, t(9;22), which results in the *BCR/ABL* fusion gene, is the dominant negative prognostic factor in ALL [94]. Its incidence increases with age, accounting for 2–5% in children and up to 28% in adult patient cohorts [95]. The disappointing response of *BCR/ABL^{POS}* patients to standard therapy and the availability of specific inhibitors for the constitutively activated tyrosine kinase in BCR/ABL fusion proteins [96] have prompted separate trials for *BCR/ABL^{POS}* and *BCR/ABL^{NEG}* ALL, making it imperative that these patients are recognized accurately. It is important to differentiate between *BCR/ABL^{POS}* ALL and BCR/ABL1-like (or Ph-like) ALL (18,19), which will be discussed later.

Cytogenetic analyses in lymphoblasts are hampered by suboptimal chromosome structure [97], and, furthermore, approximately 10% of *BCR/ABL^{POS}* ALL lack evidence of the t(9;22) by chromosome banding [95]. Both karyotyping and molecular analyses by PCR require time, 1–2 weeks and, in most laboratories, at a minimum, 24 h, respectively. The first alleged antigenic signature for *BCR/ABL^{POS}* ALL relied solely on differences in staining intensity of antigens commonly found in B-lineage ALL, such as strong expression of CD10 and CD34 and weak expression of CD38 [98–100]. However, in 1997, a preliminary analysis of 144 patients enrolled in ECOG's phase III adult ALL trial, E2993, demonstrated an association between *BCR/ABL* positivity and expression of CD25, the α-chain of the interleukin-2 receptor [101]. This observation confirmed an earlier report of the incidental finding of CD25 in four patients with *BCR/ABL^{POS}* ALL [102]. The final analysis of E2993 [31] solidified the surrogate marker profile for *BCR/ABL^{POS}* lymphoblasts as CD25^{pos}CD34^{high} with frequent dual expression of myeloid antigens, CD33 and CD13. Despite the frequent expression of two myeloid antigens by *BCR/ABL^{POS}* lymphoblasts, this phenotype must not be considered biphenotypic, since *BCR/ABL^{POS}* lymphoblasts unequivocally belong to the B-cell lineage (cCD22^{POS}, myeloperoxidase^{NEG}). Approximately 60% of *BCR/ABL^{POS}* ALL cases (pediatric and adult) express additional cytogenetic aberrations [103, 104]. Rieder et al. [105] and Primo

et al. [106] reported that patients with del(9)(p21), in addition to the t(9;22), lacked both CD33 and CD13. Among 11/156 *BCR/ABL*^{POS} patients with del(9)(p11), del(9)(del(9)(p13), or del(9)(p21) on E2993, 8 lacked both CD33 and CD13, whereas the other 3 only showed decrease or loss of CD13 expression, thus supporting and expanding Primo's observation (Paietta E, personal observation). Wetzler et al. [104] could not confirm this association between myeloid marker expression and abnormalities of 9p. Of potential interest, a report from the pre-tyrosine kinase inhibitor (TKI) era indicated that *BCR/ABL*^{POS} children with loss of the p-arm had a particularly poor outcome [103], confirming an early report from adult *BCR/ABL*^{POS} patients [105].

The dual expression of myeloid antigens, CD33 and CD13 by *BCR/ABL*^{POS} lymphoblasts, is not surprising, given that these blasts most commonly express immunophenotypic features of early pre-B-ALL (CD10^{POS}). Ludwig et al. [107] have proposed that the expression of myeloid antigens in adult B-lineage ALL differs according to the level of B-lymphoblast maturation, analogous to what has been reported from pediatric ALL [108, 109]. Analysis of E2993 [110] confirmed but also expanded these associations. While pro/pre-pre-B-ALL, the immature CD10^{NEG} maturation stage, typically expressed $CD65_{(S)}$ and $CD15_{(S)}$, the CD10^{POS} early pre-B stage preferentially expressed the pan-myeloid antigens CD33 and CD13, which in normal myelopoiesis appear before $CD65_{(S)}$/$CD15_{(S)}$ on maturing myeloid cells. However, these relationships did not hold up in *BCR/ABL*^{POS} cases; as given in Table 15.2, the paired expression of CD33/CD13 persisted irrespective of the maturation stage of BCR/ABL^{POS} lymphoblasts. CD33/CD13 positivity was seen whether *BCR/ABL*^{POS} blasts lacked CD10 (pro/pre-pre-B stage) or expressed intracytoplasmic μ chains (pre-B stage). Importantly, *BCR/ABL*^{POS} lymphoblasts never expressed $CD65_{(S)}$/$CD15_{(S)}$ even in those instances in which CD33 and/or CD13 were not expressed. It is important to realize that *BCR/ABL*^{POS} cases with CD33/CD13 expression do not represent MPAL B/myeloid, as defined by the WHO classification [9], given that *BCR/ABL*^{POS} ALL cells invariably contain intracytoplasmic CD22 (B-lineage-specific marker) and lack myeloperoxidase (myeloid-lineage-specific marker).

Table 15.2 *BCR/ABL*^{POS} B-lineage ALL blasts frequently express both CD33 and CD13, irrespective of the maturation stage of the B-lymphoblasts (shown in **bold**)

BCR/ABL	B-ALL maturation	CD33	CD13	CD65	CD15
Negative	Pro-B /Pre-Pre-B	neg	neg	pos	pos
Positive	Pro-B/ Pre-Pre-B	**pos**	**pos**	neg	neg
Negative	Early Pre-B	*pos*	*pos*	neg	neg
Positive	Early Pre-B	**pos**	**pos**	neg	neg
Negative	Pre-B	neg	neg	neg	neg
Positive	Pre-B	**pos**	**pos**	neg	neg

While *BCR/ABL*^{NEG} immature, CD10^{NEG} pro-B/pre-pre-B blasts commonly express CD65 and/or CD15, *BCR/ABL*^{POS} pro-B/pre-pre-B blasts express CD33/CD13. The more mature pre-B blasts in *BCR/ABL*^{NEG} ALL typically lack myeloid antigen expression

The (9;22)(q34;q11) translocation results in the actual Philadelphia chromosome, the derivative chromosome 22, in which the *BCR/ABL* fusion gene is located, and the derivative chromosome 9, where the reciprocal *ABL/BCR* fusion gene resides, which does not appear to contribute to the pathogenesis of this disease. Various isoforms of the *BCR/ABL* fusion gene are created dependent on the variable breakpoints in the *BCR* gene. All translated BCR/ABL proteins share a similar carboxy-terminal ABL tyrosine kinase domain (TKD), but differ in the portion of the BCR protein included in the fusion product, due to multiple breakpoint cluster regions in the *BCR* gene. In the majority of chronic myeloid leukemia (CML) cases and in one-third of *BCR/ABL*^{POS} ALL, the break within the *BCR* gene occurs in the major breakpoint cluster region (M-bcr), resulting, when joined with a portion of *c-ABL* from chromosome 9, in a *e13a2 (b2a2)* or *e14a2 (b3a2)* fusion transcript encoding a protein of 210,000 Da molecular weight (p210$^{BCR-ABL}$). A break in the minor breakpoint cluster region (m-bcr) forms the *e1a2* transcript encoding a 190,000 Da protein (p190$^{BCR-ABL}$), found mostly in *BCR/ABL*^{POS} ALL [111, 112]. Castor et al. [113] proposed a distict pattern of hematopoietic stem cell and committed B-cell progenitor involvement for major and minor *BCR/ABL* fusion ALLs, respectively.

The most striking observation with respect to CD25 and *BCR/ABL*^{POS} ALL is that expression levels of CD25 are of prognostic significance predicting for a lower likelihood to achieve complete remission [31], and shorter overall (OS) [31] or event-free survival [114] among *BCR/ABL*^{POS} patients. In other words, CD25 is unique in its dual function as a dependable marker of *BCR/ABL*^{POS} ALL and an independent prognostic factor for outcome in this disease. Most recently, the mechanism of action of CD25 in *BCR/ABL*^{POS} ALL was elucidated in detail [115]. The group of Markus Müschen [115] found that CD25 is a critical feedback regulator of the B-cell receptor (BCR) and a biomarker of tumor clones driven by oncogenic BCR mimics, including *BCR/ABL* and other *ABL1* fusion genes present in Ph-like ALL cases. This predicts that CD25-expressing B-cell malignancies may be sensitive to small-molecule inhibitors of the BCR signaling pathway (e.g., ibrutinib). In murine models for B-cell tumors, CD25 was crucial for the initiation of B-cell leukemia. Phosphorylation of the cytoplasmic tail of CD25 by protein kinase C δ (PKCδ) causes surface expression of CD25 which in turn coordinates a negative feedback by shuttling inhibitory phosphatases from the cytoplasm to the cell membrane (e.g., SHIP1). This recruitment of phosphatases stabilizes oncogenic tyrosine kinase signaling and mediates drug resistance. The mechanism of CD25 in BCR-driven lymphoid cells is illustrated in Fig. 15.3. Most importantly, CD25 inhibition sensitized CD25-expressing cells to conventional drug treatment. These preliminary data have

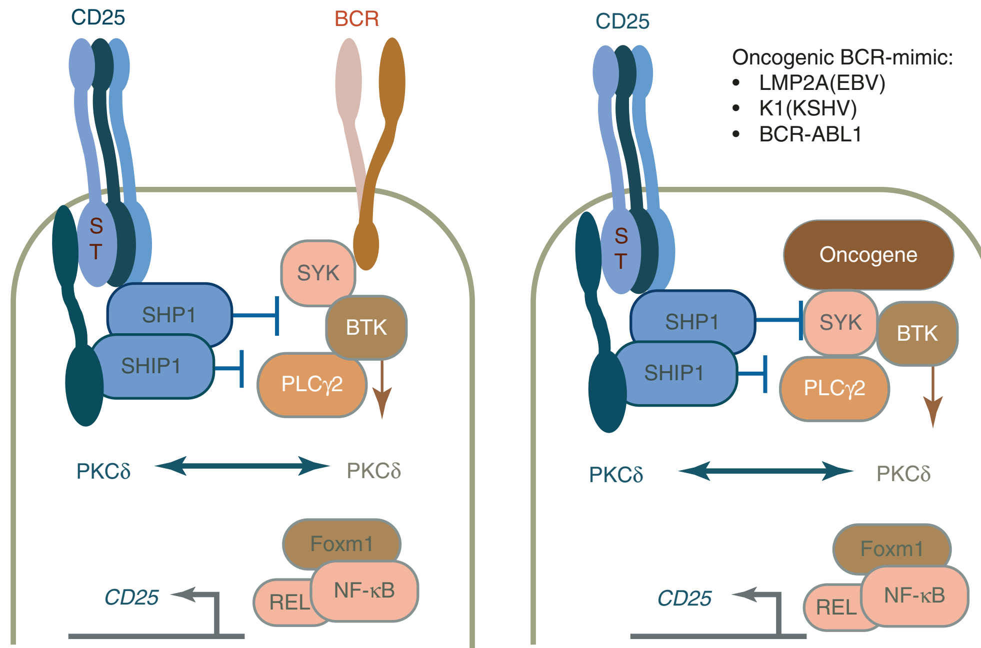

Fig. 15.3 *Left graph*: The left graph represents pre-B-cell receptor (BCR)-mediated signaling in normal B-cells. BCR signaling involves rapid activation of spleen tyrosine kinase (SYK), Bruton's tyrosine kinase (BTK), and phospholipase C γ-2 (PLCg2), which convert protein kinase C δ (PKCδ) from an inactive (*yellow*) to an activation state through phosphorylation (*blue*). Activated PCKδ (*blue*) is translocated from the cytoplasm to the plasma membrane. BCR also activates the proto-oncogene Foxm1, which encodes Forkhead box protein M1, and nuclear factor kappa B (NF-κB), a transcription factor which resides in the cytoplasm in an inactive complex. Müschen et al. have found that the genomic region of CD25 was occupied by several components of NF-κB, such as RELA and RELB, and able to interact with Foxm1. Once activated, NF-κB, and potentially also Foxm1, transcriptionally increases CD25 expression. Plasma membrane-located PKCδ (blue) recruits the newly synthesized CD25 to the cell surface via phosphorylation of the serine and threonine (ST) motif appended to the CD25 cytoplasmic tail. The now surface-located CD25 recruits phosphatases, such as Src homology region 2 domain-containing phosphatase-1 (SHP1) and phosphatidylinositol-3,4,5-triphosphate 5-phosphatase 1 (SHIP1), which counteract and stabilize BCR signaling. In normal pre-B- or B-cells, BCR signaling is transient and must be quelled by phosphatases such as SHP1 and SHIP1. *Right graph*: The right graph represents an ALL blast. ALL typically originates from pre-B-cells which critically depend on survival signals emanating from a functional pre-B-cell receptor (BCR). Oncogenes such as Epstein-Barr virus latent membrane protein 2A (LMP2A), the K1 protein of Kaposi's sarcoma-associated herpesvirus (K1), or BCR-ABL1 deregulate B-cell signal transduction by mimicking activated BCR. This "false" BCR signaling leads to constitutive activation of SYK, BTK, PLCg2, and NF-κB, which requires upregulation of surface CD25 expression via activation of PKCδ to stabilize signaling strength. With BCR mimics, BCR signaling is constitutive and excessive, a critical factor in the development of BCR-addicted leukemia and lymphoma (courtesy Dr. Markus Müschen)

led to a clinical trial using of ADCT-301, a human monoclonal antibody to CD25, linked to a pyrrolobenzodiazepine dimer toxin, in relapsed/refractory CD25^{POS} acute leukemias [NCT02588092]. As will be discussed later, CD25 also represents a most powerful prognostic factor in AML [32].

Though isolated case reports of *BCR/ABL*^{POS} ALL with T-lineage phenotype are still occasionally posted, all of the larger studies have reported that BCR/ABL rearrangements in pediatric and adult ALL are restricted to the B-cell lineage [31, 96, 116–118]. A substantial cohort of pediatric patients studied from various European groups found 3 T-ALLs among 61 *BCR/ABL*^{POS} patients [119]; unfortunately, no details were provided on the immunophenotypic diagnosis of those T-lineage cases, though it is apparent that cCD3 and cCD22 were not tested. The lack of demonstration of these lineage-specific antigens is the most likely reason for misdiagnosed *BCR/ABL*^{POS} T-cell phenotypes. A potential alternative cause is an apparent Philadelphia chromosome translocation by conventional karyotyping that does not yield the ABL-BCR juxtaposition on chromosome 9 [120]. While in the report by Fossa et al. [120] the leukemic phenotype was unequivocally T-lymphoid (cCD3^{POS}), an analogous case was seen in ECOG's E2993 trial, with a (9;22)(q34;q11.2) translocation that by FISH did not result in the *BCR/ABL* fusion but, as in Fossa's case, disrupted the *BCR* gene (ECOG Cytogenetics Committee, personal communication). The E2993 patient, however, expressed immunophenotypic features of pre-B-ALL. In summary, caution is definitely warranted when t(9;22)^{POS} or *BCR/ABL*^{POS} T-ALL cases are published.

t(4;11)(q22;q23)-*KMT2A (former MLL)/AF4*^{pos} B-Lineage ALL

The myeloid/lymphoid or mixed-lineage leukemia (*MLL*) gene has been renamed *KMT2A* gene, located at 11q23. *KMT2A* in humans encodes histone-lysine N-methyltransferase 2A. *KMT2A/AF4*^{POS} ALL is typically found in early infancy

and also represents the most prevalent of the diverse *KMT2A* fusion genes in adult ALL [121–123]. Despite improved complete remission rates with modern treatments, overall survival in adults remains poor due to short remission duration [122–125]. There exists a striking difference in outcome by age group with the worst prognosis found in infants less than 6 months old as well as in adults [124]. *KMT2A/AF4*^{POS} ALL is associated with the immature pro/pre-pre-B-ALL maturation stage, defined by negativity for CD10. As a unique immunophenotypic feature among all ALL phenotypes, *KMT2A/AF4*^{POS} lymphoblasts show a tendency to express the more mature myeloid carbohydrate antigens, $CD65_{(S)}$ and $CD15_{(S)}$, while lacking CD33 and CD13, the pan-myeloid antigens expressed earlier in normal myelopoiesis [93, 123, 125, 126]. Overexpression of immature antigens, CD133 and CD135, further supports the immature state of this subtype [125]. On the other hand, CD33 and/or CD13 are found preferentially in CD10^{pos} early pre-B-ALL. Burmeister et al. [126] demonstrated that lymphoblasts from *KMT2*-rearranged adult ALL patients in general (55% with *KMT2A/AF4*) demonstrated significantly lower expression of CD10, CD33, and CD13 and more frequent expression of $CD65_{(S)}$ and $CD15_{(S)}$ irrespective of B-lineage differentiation stage. This report confirmed the observations from the E2993/UKALLXII trial [125]. Together with the myeloid antigen expression pattern in *BCR/ABL*^{POS}ALL, discussed before, these observations suggest that myeloid antigen expression in B-lineage ALL is determined by the underlying genetic defect rather than B-lymphoid developmental stage.

Cryptic t(12;21)(p13;q22)-*TEL(ETV6)/AML1(RUNX1)*^{POS} B-Lineage ALL

The cryptic (12;21)(p13;q22) translocation that results in the *TEL/AML1* fusion gene is the most common genetic aberration in pediatric ALL (>20%) and carries a favorable prognosis, especially with high-intensity therapy [127, 128]. In adults, this hybrid gene only accounts for <1–3% of ALL [95, 100, 129], thus precluding prognostic predictions. In children, the *TEL/AML1* fusion occurs exclusively in early pre-B-ALL with certain characteristic markers: high intensity of HLA-DR, CD40, and CD10 expression (early pre-B phenotype), commonly CD13^{POS} and/or CD33^{POS}, but CD9^{NEG}, CD20^{NEG}, and low expression of CD45, CD135, and CD86 [130–132]. A striking characteristic feature has recently been added, namely absence of CD11b, an integrin reported to confer poor response to therapy in B-lineage ALL [133]. Given the low incidence of *TEL/AML1*^{POS} ALL in adults, immunophenotypic information in this age group is scarce. The nine *TEL/AML1*^{POS} cases found among ECOG E2993 patients (1.4%) demonstrated the characteristic CD10^{POS} early pre-B phenotype, lacking CD20 and predominantly CD34, and frequently expressing CD33+CD13. However, in comparison to early pre-B-ALL with normal cytogenetics and molecularly negative for *TEL/AML1, BCR/ABL, MLL/AF4, and E2A/PBX1*, there was no difference in CD45, and the CD40 intensity was higher than the median value in normal karyotype controls in only half of the patients (Paietta E, unpublished observation). Although based on small numbers of patients, the most predictive immune profile applicable for both pediatric and adult *TEL/AML1*^{POS} B-lineage ALL, to date, is CD10^{POS}, CD20^{NEG}, CD34^{NEG}, cIgM^{NEG}, frequently CD33+CD13^{POS}, and possibly CD11b^{NEG}.

Inv(7)(p15q34) and t(7;7)(p15;q34)-*TRB@/HOXA*

As a result of two cryptic cytogenetic aberrations, inv(7)(p15q34) or t(7;7)(p15;q34), the TCRB locus (*TRB@*) at 7q34 is juxtaposed to the *HOXA@* at 7p15 (approximately <5% of pediatric and adult T-ALL), leading to transcriptional activation of several *HOXA* genes [134, 135]. The typical antigen expression patterns associated with the *TRB@/HOXA* rearrangement include negativity for CD2 and single expression of CD4, without CD8. It is important to emphasize that among surface antigens, CD7 is the only T-cell antigen universally expressed by T-cell lymphoblasts.

Antigens and Antigen Profiles Associated with Prognostic Gene Expression

FLT3 Mutations in AML

The *FLT3* gene encodes CD135, the FMS-like tyrosine kinase 3 (FLT-3) or receptor-type tyrosine-protein kinase FLT3, a cytokine receptor which belongs to the type 3 receptor tyrosine kinases and is expressed on the surface of normal hematopoietic progenitors and the leukemic blasts of most cases of AML. Upon binding of the FLT3 ligand, the receptor undergoes dimerization and subsequent autophosphorylation of the tyrosine kinase domains, leading to the induction of multiple intracellular signaling pathways involved in cell growth, differentiation, and survival [136]. There are two major clusters of mutations of the FMS-like tyrosine kinase-3 (*FLT3*) gene, those in the juxtamembrane domain that lead to internal tandem duplications (ITD) and point mutations in the tyrosine kinase domain (TKD. Both result in the activation of the transforming potential of *FLT3* [136, 137]. Activating mutations of the *FLT3* gene are the most common known genetic abnormalities in pediatric and adult AML, with *FLT3-ITD* found in approximately 20–35% of adults and 15% in children (ranging from 1.5% in infants to 20% at teenage age), while *FLT3-TKD* are present in about 7% of AML irrespective of age group. With respect to cytogenetic links, distinct differences are seen between *FLT3-ITDs* and *FLT3-TKDs*; *FLT3-ITDs* are particularly frequent in AML with normal cytogenetics, while *FLT3-TKDs* frequently occur in cases with inv(16)-*CBFβ/MYH11*. Both types of mutations are found in a large percentage of t(15;17)-*PML/RARα* APL with minimal impact on outcome, if treated according to modern strategies [138, 139]. In non-APL AML, the suggested negative clinical implications for *FLT3-*

ITD (e.g., increased relapse rate) vary with therapeutic intensities, allelic ratio (mutational burden), and size of ITD, and according to its genetic context [140, 141]. *FLT3-TKDs*, on the other hand, failed to confer unfavorable prognosis [142]; in fact, patients with high-level mutations (more than 25% mutant) experienced improved outcome [143]. The introduction of novel FLT3 inhibitors will certainly change the prognostic outlook of patients with FLT3 gene alterations [144].

The earliest description of a unique immunophenotype for *FLT3-ITD*^{POS} AML suggested a combination of increased CD123, decreased CD38, "mildly" decreased CD117, and loss of CD133 [145]. Subsequently, the same authors described the distinctive, cuplike nuclear morphology in *FLT3-ITD*^{POS} AML (which was discussed before) in association with loss of HLA-DR, CD34, and CD133 [73]. Falini et al. reported this paired absence of CD34/CD133 in *NPM1*-mutated AML in their groundbreaking publication in 2005 [146]. The frequent association of *FLT3-ITD* with *NPM1* mutations [114, 140, 141], therefore, suggests that the lack of CD34 and CD133 in *FLT3-ITD*^{POS} AML may be the result of the concomitant presence of mutated *NPM1*. Gönen et al. [32] reported that CD123 expression, but not CD34 or CD133, was significantly higher in CD25^{POS} AML which demonstrated a characteristic genotype of triple *FLT3-ITD/NPM1/DNMT3A* mutations. Co-association of *FLT3-ITD* with *DNMT3A* and *NPM1* mutations is well established [14, 147]. In mice, concurrent expression of *FLT3-ITD*, mutated *DNMT3A*, and mutated *NPM1* resulted in a fully penetrant leukemic phenotype, whereas any single or paired alleles led to incompletely penetrant disease [148]. Angelini et al. [149] also reported the combined expression of CD123 and CD25 as characteristic for *FLT3-ITD*^{POS} AML and, added CD99 (MIC-), a potential LSC marker, highly expressed in the LSC-enriched CD34^{POS}/CD38^{NEG} fraction compared to the bulk of blasts [150]. CD25^{POS} *FLT3-ITD*^{POS} AML patients on phase 3 trial E1900 [NCT00049517] had a significantly shorter overall survival than CD25^{NEG} FLT3-ITD^{POS} patients, whose survival did not differ from that of the *FLT3*-wild-type cohort [32].

FLT3-ITD^{POS} T-Lineage ALL

The rare subtype of CD117^{POS} T-lineage ALL with activating *FLT3* gene mutations [65, 151] belongs to the few known examples of gene mutations, which are predicted by the presence of a unique antigen expression pattern.

In pediatric ALL, *FLT3-TKDs* are common in cases with *KMT2A/MLL* rearrangements and those with hyperdiploid karyotype, while *FLT3* length mutations are extremely rare; still, high levels of FLT3 protein expression provide a target for therapeutic intervention [136, 140, 152, 153]. While in infant *KMT2A/MLL*-rearranged ALLs the presence of a *FLT3* mutation negatively affected outcome, there was no such effect seen in hyperdiploid cases [152]. *FLT3* mutations are even rarer in adult ALL. The analysis of ECOG study E2993 revealed an incidence of 1.9% *FLT3-ITD* among 511 patients [151]. Of the ten E2993 *FLT3-ITD*^{POS} patients, three belonged to the B- and the remaining seven to the T-cell lineage. No consistent immunophenotypic or cytogenetic features were shared by the three early pre-B patients. On the other hand, all *FLT3-ITD*^{POS} T-ALLs expressed a unique immunophenotype: CD5^{NEG}; surface CD3^{NEG}CD4^{NEG}CD8^{NEG} (triple-negative); positive for CD117, CD34, CD2, CD7, TdT, CD62L, CD13, CD135 (FLT3 protein); and positive for T-cell lineage-specific cCD3 [65]. CD117, the stem cell factor receptor, is much more frequently expressed by leukemic myeloblasts than lymphoblasts [154, 155]. In normal lymphopoiesis, a fraction of CD3/CD4/CD8-triple-negative CD34^{POS} thymocytes express high levels of CD117 [156, 157]. In these thymocytes, expression of CD117 coincides with that of CD135, the FLT3 receptor tyrosine kinase [136, 158]. Occasionally, up to 10% of cCD3^{POS}CD117^{POS}*FLT3-ITD*^{POS} blasts expressed myeloperoxidase, thus representing truly biphenotypic features [65]. This profile fits the most immature category of T-ALL [159–161], resembling earliest thymic precursors with both T- and myeloid lineage potential [162]. In further support of a T-lineage affiliation, FLT3-mutated T-lymphoblasts overexpressed *LYL1* and *LMO2* oncogenes [160]. In pediatric T-ALL, the LYL1-overexpressing, most immature cases demonstrate relative resistance to standard chemotherapy [160]. To the contrary, the *FLT3-ITD*^{POS}CD117^{POS}CD13^{POS} T-cell phenotype in adults does not carry inferior prognosis, though numbers were small [151]. This observation is remarkable given the negative prognostic impact of CD13 expression in CD117^{NEG} adult T-lineage ALL [151]. Neumann et al. [163] subsequently confirmed the CD2^{POS}/CD5^{NEG}/CD13^{POS}/CD33^{NEG} phenotype as a surrogate for mutated *FLT3* in T-cell ALL. They expanded the distinct gene expression pattern (aberrant expression of *IGFBP7*, *WT1*, *GATA3*) and mutational status (absence of *NOTCH1* mutations and a low frequency, 21%, of clonal TCR rearrangements). Despite the low frequency of *FLT3* gene mutations in adult ALL overall, the availability of a variety of FLT3-kinase inhibitors suggests a potential targeted approach in the treatment of this patient cohort. The potential of targeted therapy is the clinically important aspect in this ALL subtype and should supersede any discussions as to whether *FLT3*-mutated T-ALL represents a biphenotypic leukemia or an example for MPAL T/myeloid as defined by the WHO classification [9].

Van Vlierberghe et al. [164] reported that in pediatric T-ALL CD117 mRNA (not protein) expression was not invariably associated with *FLT3* gene mutations in that most pediatric cases expressed CD117 mRNA; this suggests that, similar to what has been reported for myeloperoxidase transcripts [165], CD117 mRNA might undergo posttranscriptional downregulation in ALL. Immunophenotypic profiles of the two pediatric *FLT3-ITD*^{POS} T-ALL patients identified by Van Vlierberghe et al. [164] were surface CD3^{NEG} but CD5^{POS}CD4^{POS} with partial CD13 expression. Furthermore, the authors stated that these blasts carried a "*HOX11L2*

translocation," presumably leading to aberrant HOX11L2 expression. Taken together, these findings suggest arrest at an immature, though already single, CD4^{pos} differentiation level, distinct from the triple-negative stage seen in the adult cases [65]. Remarkably, another pediatric T-ALL case with *FLT3-ITD*, the only one among 59 children tested, identified by Ferrando et al. [160] belonged to the LYL^{POS} cluster, consistent with the findings in the adult cases.

Hoehn et al. concluded that the absence of CD117 expression in T-ALL precluded the presence of *FLT3* mutations, whereas the positive predictive value of CD117 for mutated *FLT3* was only 35% [166]. CD117^{pos} T-ALL lacking *FLT3* gene mutations were found in 2% of patients on E2993 [93]. Aside from CD117, their immunophenotype differed from that in *FLT3-ITD*^{POS} cases subtly but distinctly: the T-lymphoblasts expressed CD5, occasionally were single CD8^{POS}, frequently expressed CD33 instead of CD13, often were CD56^{POS}, and commonly lacked CD34 and CD62L, both markers of immaturity. A close association between CD117 and CD13 expression in surface CD3^{NEG} T-ALL has been previously reported [167]. Thus, while CD117 expression in adult T-lineage ALL might be considered a surrogate marker for *FLT3* gene mutations, this concept only applies for T-lymphoblasts arrested at the most immature stage of differentiation.

CD117^{POS}/*FLT3-ITD*^{POS} T-ALL represents a subtype of early T-cell precursor (ETP) leukemia, which, particularly in adults, has been demonstrated to be widely heterogeneous both in immunophenotype and genetic alterations [168–172].

Early T-cell Precursor (ETP) Leukemia

ETP ALL was initially characterized solely based on its immunophenotypic features, CD1a^{NEG}CD8^{NEG}CD5^{WEAK} with myeloid (e.g., CD33, CD13, CD11b, or CD65), and stem cell markers (e.g., CD34 and CD117), suggesting that this leukemia subtype may be part of a spectrum called stem-cell-like leukemias [173]. The name ETP was given to these leukemias because their immunophenotype appeared closely related to that of early T-cell precursors which retain their multilineage differentiation potential [162, 174, 175]. ETP-T-ALL accounts for approximately 10–15% of pediatric T-ALL [170] but has a much higher prevalence of 50% in adult T-ALL in some studies [168] but not others [176]. Though early data suggested that this subtype of T-ALL carried a very poor prognosis [173, 176–178], more recent results are encouraging [179–181].

Genetic alterations fueling ETP-ALL suggest that this leukemia has more in common with AML than with other types of ALL [168–170, 172, 182]. ETP-ALLs contain mutations in myeloid-specific oncogenes and tumor-suppressor genes, including *IDH1, IDH2, DNMT3A, FLT3, GATA3, ETV6*, and *NRAS.* On the other hand, ETP-ALLs showed a low incidence of alterations commonly seen in T-ALL, such as activating mutations in the *IL7R* gene and *NOTCH1*, while approximately 25% demonstrated co-occurrence of myeloid and T-typical mutations. In an analysis of ECOG trial E2993, Van Vlierberghe et al. [33] found that in these ETP-ALLs, mutations in *IDH1/IDH2* and *DNMT3A* were associated with poor prognosis. As much as ETP-ALL represents a grey zone with respect to its genetic affilations, it presents a challenge to us flow cytometrists. How many cases of ETP-ALL are indeed enrolled on AML treatment protocol because they were diagnosed as AML based on antigen profile? It has been suggested that ETP-ALL may benefit from myeloid therapy, though we will never get the answer to this if we do not establish a clear guideline for the immunophenotypic diagnosis of this subtype. I would like to suggest the following: any case with expression of surface CD7, CD2, and intracytoplasmic CD3; absence or weak surface expression of CD5; lack of surface CD3; expression of CD34, CD13, and/or CD33; and whether or not CD117 or myeloperoxidase are found should be considered ETP-ALL.

Whether or not an ETP ALL patient should be treated as ALL or AML is currently unclear. ALL COG study AALL0434 (NCT00408005) suggested that there was no difference in outcome between ETP, near-ETP, and not-ETP patients, whereby the distinction of near-ETP was based on elevated CD5 expression in otherwise ETP patients [179]. Induction failure, based on >25% blasts by morphology at the end of induction, was significantly higher in ETP and near-ETP patients. Day 29 bone marrow MRD levels of >0.01% were present in 81.4%, 64.8%, and 30.5% of ETP, near-ETP, and not-ETP patients. Day 29 MRD levels were used for risk stratification, whereby intermediate- (MRD <1%) and high-risk patients (MRD >1%) were randomized to receive or not receive nelarabine, delayed intensification, and maintenance. With this MRD-targeted approach, ETP patients had outcome identical to not-ETP patients.

However, there is the caveat when interpreting this study in that the immunophenotypic analysis for ETP-ALL status on AALL0434 was not completely assessed for all patients [183]. But can we solely rely on immunophenotypic features in defining ETP-ALL? Retrospective analyses of T-ALL samples with ETP-gene signature have not consistently been found to express the ETP-ALL immunophenotype [184, 185]. But what is the best way to identify ETP-ALL, or more importantly ETP-ALL with poor outcome? [186]. Bond et al. [187] reported that deregulation of homeobox (*HOX*) factor A was highly predictive of phenotypic immaturity, glucocorticoid resistance, and early treatment failure in T-ALL. However, while the entire *HOX*-activated group did not have an inferior outcome, poor prognosis was restricted to those patients who also had an ETP-like immunophenotype. Vice versa, ETP-ALL patients without *HOXA*-activation fared equally well as non-ETP patients. A subset analysis of AALL0434 [183], which included 10 patients who had failed induction therapy, corroborated the notion that *HOXA*-activated ETP-ALL, frequently acquired in *MLL (KMT2A)*-rearranged cases, is

associated with induction failure, refractory disease, and relapse. Unfortunately, the detection of *HOX*-activated ETP-ALL carries its own pitfalls.

To date, alternative treatment options for ETP-ALL are limited. One could foresee, however, that a precision medicine approach be suggested in the near future, e.g., using a histone methyltransferase inhibitor to target *HOX* gene expression [188]. A phase 1 trial using EPZ-5676, an inhibitor of the DOT1-like histone H3 methyltransferase, in relapsed/refractory leukemia with *KMT2A* rearrangements has recently been completed (NCT02141828). So, when the time comes and a precise definition of ETP-ALL is important for targeted therapy, the burden will be in part on the flow cytometrist, the first one who could raise a suspicion of ETP-ALL in a new case of leukemia.

Association of T-ALL Differentiation Stages with Gene Expression

The changes brought about in the classification of T-cell ALL over the last 15 years have been dramatic, considering that where we started from was a mere distinction between CD2^{POS} and CD2^{NEG} T-ALL. In 2002, Ferrando et al. published gene expression signatures in pediatric T-ALLs which correlated with leukemic arrest at specific stages of normal thymocyte differentiation [160]. This elegant model was based on the unique, aberrant expression of oncogenic transcription factors in T-ALLs as a result of chromosomal translocations which typically juxtapose strong promoter and enhancer elements associated with *TCR* genes to a finite number of T-cell transcription factors. This T-ALL classification distinguished the early immature double-negative CD4^{NEG}CD8^{NEG}surface CD3^{NEG} stage with overexpression of transcription factor *LYL1* and *LMO2*, which corresponds to ETP-ALL with a transcriptional program related to hematopietic stem cells and specific genetic aberrations, as previously discussed. In contrast, the early cortical stage is characteristically CD1a^{POS} CD4^{POS}CD8^{POS}CD3^{WEAK}CD10^{POS} and frequently associated with cytogenetic translocations inducing activation of the *TLX1/HOX11* family of homeobox transcription factor oncogenes. Finally, the late cortical CD4^{POS}CD8^{POS}CD3^{HIGH} CD2^{POS}TCR^{POS} stage shows expression of the TAL1 transcription factor oncogenes. All of these stages precede that of mature single positive with either surface CD4 or CD8 expression. Constitutive expression of *TLX1*/*HOX11* has been associated with favorable prognosis in pediatric and adult T-lineage ALL [160, 189–191]. In the analysis of a large series of adult T-ALL enrolled on E2993 [151] all cases were tested for cCD3 and myeloperoxidase. Aside from cCD3, only surface CD7 was universally present. Attempts to stratify patients according to their maturation stage based on expression of CD3, CD2, or CD34, as suggested by the WHO classification system [9], failed to provide prognostic subsets. While the myeloid antigens CD13 and/or CD33 were found in approximately half of the patients, the carbohydrate antigens, CD65$_{(S)}$ and CD15$_{(S)}$, were detected in a minority of cases. The entire patient cohort could be divided into two prognostic subsets based on CD1a and CD13 expression: CD1a^{POS} T-ALL predominantly lacked CD34, CD13/CD33, and CD11b. These T-lymphoblasts, representative of intrathymic differentiation, frequently expressed the CD62L selectin and CD10, and were double positive for CD4 and CD8. CD1a^{POS} T-ALL did exceedingly well with an overall survival at 5 years of 64% (95% CI 48–80%). The other subtype with clinical significance was CD13^{POS} T-ALL. While the complete remission rate was similar to that of CD13^{NEG} patients, overall survival at 5 years was significantly shorter than for CD13^{NEG} patients ($p = 0.0005$). CD13^{POS} or double-CD13/CD33^{POS} T-lymphoblasts preferentially expressed CD34 and CD11b and showed a tendency to be negative for CD4, CD8, surface CD3, or CD2. CD117^{POS} T-ALL was diagnosed in 18% of patients. Only 6/107 patients with unequivocal staining for cCD3 and negativity for cCD22 expressed CD19, occasionally in combination with CD10. Of potential interest, merely 17% of these patients survived for 5 years. In a follow-up analysis, Van Vlierberghe et al. [33] performed microarray gene expression profiling of these immunophenotypically characterized patients and described two clusters. The first corresponded to early immature leukemia (ETP) with a gene expression signature related to that of hematopoietic stem cells and myeloid progenitors. In contrast, the second cluster contained leukemias with gene expression signatures of cortical and mature thymocytes. Overall, the immature cohort was associated with poor prognosis and reduced overall survival compared with cortical/mature T-ALL ($p = 0.0112$). Notably, the poor prognostic effect of CD13 expression was restricted to the cortical/mature T-ALL, thus identifying a high-risk subgroup in an otherwise good prognosis subtype.

NPM1-Mutated AML

The *NPM1*-mutated AML subtype is characterized by high frequency of *FLT3-ITD*, association with normal karyotype, negativity for both CD34 and CD133, and good prognosis, especially in the absence of *FLT3ITD* [75]. Other reports have suggested additions and/or modifications to this phenotype. Nomdedeu et al. [192] concluded that the majority of cases showed high expression of CD33, CD13, HLA-DR, and CD123, and myelomonocytic traits in the morphologic appearance. On the other hand, Kern et al. [193] suggested high expression of myeloperoxidase and CD33 but lack of CD13, CD65, CD15, HLA-DR, and CD34, combined with minimal differentiation in 20 cases of *NPM1*-mutated AML. The rare immunophwnotypic subset of *NPM1*-mutated/*FLT3-ITD*^{POS} normal karyotype AML with dual-CD7/CD19 expression [58] presents with surprisingly high CD34 expression, higher than

found in *NPM1*-mutated/*FLT3-ITD*^{POS}AML without this antigen profile or *NPM1*-mutated AML without *FLT3-ITD*. An informal analysis of the incidence of CD34^{POS} myeloblasts in 105 cases of *NPM1*-mutated AMLs suggested greater variability than previously appreciated with the percentage of CD34^{POS} blasts ranging from 0 to 99% (Paietta et al., unpublished observation). Taken together, these reports suggest that the immuneprofile of *NPM1*-mutated AML is not as homogenous as previously thought. Along the same line, LICs in *NPM1*-mutated AML have been located both in CD34^{POS} and CD34^{NEG} stem cell fractions [7]. Martelli et al. [194] reported that CD34^{POS} cells from *NPM1*-mutated cases harbored aberrant nucleophospmin expression in the cytoplasm. The *NPM1*-mutated gene and/or protein was also confirmed in the CD34^{POS} subfraction which expressed a stem cell-like phenotype, CD34^{POS}, CD38^{NEG}, CD123^{POS}, CD33^{POS}, and CD90^{NEG}. When transplanted into immunecompromised mice, these CD34^{POS} cells generated a leukemia indistinguishable from the original disease with *NPM1*-mutated CD34^{NEG} myeloblasts, suggesting that the CD34^{POS} cell fraction in *NPM1*-mutated leukemia contains cells with LIC properties.

BCR/ABL1-like or Ph-like B-lineage ALL

Just like T-lineage ALL, B-lineage ALL has undergone a major reclassification with strong prognostic and therapeutic implications. FAB classification of ALL has become obsolete [9, 47]. The standard subclassification of B-lineage ALL based on maturation stages lacks prognostic significance with modern chemotherapy [195]. The associations between level of maturation and major genetic subtype in B-lineage ALL have been discussed earlier for the historic genetic defects, *BCR/ABL, MLL/AF4,* and *TEL/AML1*. *E2A/PBX1* (now called *TCF3/PBX1*) transcripts, derived from the (1;19)(q23;p13) translocation, are more commonly associated with more mature pre-B ALL, characterized by the presence of intracytoplasmic IgM heavy chains. This subtype was previously considered as high risk, but is no longer included into the risk stratification as a result of modern treatment regimens [196]. It is difficult to distinguish mature B-ALL from the leukemic phase of Burkitt's lymphoma, as they share immunophenotypic features, clinical presentation, and cytogenetic abnormalities. In the prototype (8;14)(q24;q32) translocation, the c-myc proto-oncogene is translocated from chromosome 8 onto chromosome 14 and brought under the transcriptional control of the immunoglobulin heavy-chain locus. The variant translocations, t(2;8)(p12;q24) and t(8;22)(q24;q11), result in the positioning of portions of the κ and λ light chains, respectively, under the control of myc.

The revolution in B-lineage ALL classification started with the finding of alterations of the lymphoid transcription factor gene *IKZF1* (IKAROS) in B-ALL [170, 182]. *IKZF1* deletions and somatic mutations are the hallmark of *BCR/ABL*^{POS} ALL and confer poor prognosis in this disease [197–199]. Recently, Iacobucci et al. described surface expression of CD90 as a marker for *IKZF1* alterations in *BCR/ABL*^{POS} ALL [200]. CD90/Thy1 is a marker of hematopoietic stem cells [201]. In combination with surface CD25 expression [17, 31, 101], CD90 analysis affords the flow cytometrist to reliably predict the presence of *BCR/ABL* transcripts in B-ALL. As we discuss below, it is essential to exclude CRLF2-overexpressing *BCR/ABL1*-like ALL, which can present with both CD90 and CD25 expression [Paietta E, unpublished observation]. Noteworthy, however, neither CD25 nor CD90 will be expressed in a small percentage of *BCR/ABL*^{POS} patients, those with superior outcome [31]. The same is true for other immunophenotypic features published as associated with *BCR/ABL*^{POS} ALL, such as high CD34 and low CD38 expression and dual expression of CD13 and CD33 [98–100]. While in a case with B-lineage ALL, an immune profile with expression of both CD25 and CD90 and absence of CRLF2 expression, is highly predictive for the presence of *BCR/ABL* transcripts, I always advise to run the *BCR/ABL* PCR assay to confirm the immunophenotypic findings, if at all possible.

IKZF1 can also be deleted in *BCR/ABL*^{NEG} B-ALL, likewise associated with inferior outcome [199, 202]. Many of these *BCR/ABL*^{NEG}, *IKZF1*-mutated cases exhibit a gene expression profile similar to that of *BCR/ABL*^{POS} ALL with novel kinase-activating mutations (e.g., *JAK2*, *ABL1, PDGFRB, CSF1R*) or genetic alterations in signaling pathways (e.g., *EPOR, IL7R, SH2B3*), giving birth to the novel subtype of *BCR/ABL1*-like ALL (Ph-like ALL), which is incorporated into recent WHO revisions as a provisional category [13]. With continuously extended sequencing, the full repertoire of kinase-activating lesions and other rearrangements in Ph-like ALL awaits to be identified. Ph-like ALL has been characterized independently by two groups [199, 203]. Characteristically, these patients show high levels of MRD after induction and overall poor outcome. MRD-guided, risk-directed therapy, including allogeneic transplantation, may be able to abolish the adverse prognostic significance of Ph-like ALL, at least in children [204]. However, a recent report from German Multicenter ALL Working Group (GMALL) trials 06/99 and 07/03 suggested that adult Ph-like ALL patients, although they achieve complete hematologic remission (CR), relapse very rapidly after induction therapy, thus precluding stem cell transplantation in first CR, as had been stipulated in the protocols [205]. Fortunately, there are exciting genomic and preclinical findings which suggest that many patients with Ph-like ALL could be successfully treated with currently available lesion-specific kinase inhibitors [170, 182, 206, 207]. Tasian et al. [207] demonstrated the need for combinatorial treatment strategies which hit or ablate an entire multifaceted pathway affected in these patients using patient-derived xenograft models. About 15% of children present with Ph-like ALL and, just like with *BCR/ABL*^{POS} ALL, the incidence of Ph-like

ALL showed increase with age in a large study across various National Cancer Treatment Network groups in the USA [19]. In contrast, GMALL investigators reported the peak of Ph-like ALL among adolescents and young adults and a dramatic decrease in the incidence of Ph-like ALL with more advanced age [208].

Of utmost relevance to flow cytometrists, approximately 50% of Ph-like ALL patients harbor a rearrangement of *CRLF2* (cytokine receptor-like factor 2 or thymic stromal lymphopoietin receptor), either as an *IGH@-CRLF2* rearrangement or a focal deletion proximal to *CRLF2* that results in expression of the *P2RY8-CRLF2* fusion, both causing overexpression of *CRLF2* on the surface of the leukemic cells [209, 210]. Half of *CRLF2*-rearranged cases harbor concomitant activating *JAK1/2* mutations [209–211], and *CRLF2*-rearranged cases exhibit constitutively active JAK-STAT signaling and activation of the P13K/mTOR pathway making them sensitive to JAK mTOR inhibitors [170]. It is important to realize that CRLF2 overexpression occurs only in B-ALL cases which lack rearrangements of *TEL, MLL (KMT2A), TCF3*, and *BCR/ABL*. Rare cases of *CRLF2* overexpressing ALL patients without the molecular features of the Ph-like phenotype have been seen predominantly in children (Roberts K, personal communication). There are inconsistent data on the prognosis of *CRLF2*-rearranged Ph-like ALL cases [30, 212–214]. Whatever the prognostic power of CRLF2 overexpression, these patients may be treated with biologic treatment strategies and spared a stem cell transplant. Thus, the recognition of these cases is important and it is possible using a monoclonal antibody to CRLF2. This antibody must be included into any antibody panel used routinely by clinical laboratories in the evaluation of B-cell leukemias.

Antigens and Therapy

Acute leukemias provide a suitable testing environment for therapy with antibodies, which represent drugs with a clearly defined target, the specific antigen. There are several aspects to antigens and antibodies and their usefulness in therapy. Selected expression of antigens by leukemic cells is a first prerequisite. In the majority of cases, antibodies to antigens expressed by the leukemic cells are administered in in vivo treatment. Recently, adoptive immunotherapy with autologous chimeric antigen receptor-engineered T-cells (CAR-T-cells) has become a major focus of leukemia therapy, particularly in ALL. Antibodies are being incorporated into first-line therapies, to eliminate MRD, or during salvage attempts in relapsed/refractory patients [215–221]. Undoubtedly, with the documented benefits of antibody therapy, particularly in ALL, and ongoing optimization of CAR-T-cell therapy, antigen-specific immunotherapy strongly contributes to targeted or personalized therapy, thereby changing the paradigm of treatment of the acute leukemias.

Treatment with Monoclonal Antibodies

In vivo therapy with monoclonal antibodies aims at a specific antigen, which otherwise may be a part of a very diverse immunophenotype. Targeted delivery of these agents based on recognition of their relevant antigen on the surface of the leukemic cells improves efficacy and, optimally, minimizes off-target toxicity, provided that antigen distribution in normal tissues is well known (cytotoxic effects may be more widespread than intended). While there is a plethora of potential antibody targets on leukemic cells, only a select few have emerged as clinically successful. Reasons are multiple and include specificity of antigen expression on target tissue (i.e., the leukemic cell) and the question whether the target antigen is expressed by the LIC in individual patients. Monoclonal antibodies can be used naked, in unconjugated form (e.g., rituximab) and exert their effect via various mechanisms, including antibody-dependent or complement-dependent cytotoxicity or induction of apoptosis. Alternatively, conjugated antibodies are used, whereby they function as vehicles carrying immunotoxins or chemotherapeutic agents (e.g., calicheamicin-conjugated CD33 antibody), which requires knowledge that the target is internalized upon binding of the antibody [222]. Radioimmunotherapy, which adds radiobiological cytotoxicity to immunologic cytotoxicity by using monoclonal antibodies (e.g., CD45, CD33, CD22) conjugated to radioactive molecules, has been tested in AML or ALL, predominantly for its efficacy in intensifying the antileukemic effects of conditioning regimens prior to various types of stem cell transplantation [223]. T-cell engaging bi-specific antibodies use the host's cytotoxic T-cells to eliminate leukemic cells [215, 216, 219, 224].

CD20 Antibodies in B-ALL

In B-lineage ALL, CD20 expression depends on the maturation stage of malignant B-lymphoblasts with CD10^{NEG} pro-B/pre-pre-B lymphoblasts generally lacking CD2 [125], while CD20 expression by CD10^{POS} early pre-B-ALL blasts is variable [226]. But even when expressed, antigen density is usually quite low [110, 225]. Importantly, both the percentage of CD20^{POS} blasts and the intensity of staining are greatly affected by the choice of fluorochrome used in CD20 antibody binding in that CD20 antibody conjugated to fluorescein isothiocyanate (FITC) will yield much lower numbers than CD20 antibody conjugated to phycoerythrin (PE). This important information is not paid attention to in publications

regarding the prognosis of CD20 expression in B-lineage ALL or the result of rituximab treatment [215, 216, 227]. Since the report of the trial, the Group for Research on Adult Acute Lymphoblastic Leukemia 2005 (GRAALL 2005), on rituximab in *BCR/ABL*^{NEG} CD20^{POS} B-lineage ALL at the plenary session of the American Society of Hematology meeting in 2015 and recently published in the New England Journal of Medicine [228], the addition of rituximab to the treatment of ALL has been accepted as standard of care. In this seminal trial, patients with <20% CD20^{POS} blasts were excluded and only those with at least 20% CD20^{POS} blasts were assigned to rituximab treatment or not. In this setting, and giving rituximab to all treatment phases, the rituximab group showed improved event-free survival and a reduction in the cumulative incidence of relapse when compared to the control group. Of note, CR rates were identical and MRD levels post-induction were comparable in the two groups suggesting that rituximab addition to induction therapy did not improve the quality of response. This is in contrast to the results of the GMALL Study 07/2003 [229] which also showed a comparable morphologic CR rate between patients treated with rituximab and those not receiving the antibody but in which the MRD load, measured by PCR, reduced significantly faster in the rituximab group. Interestingly, Thomas et al. [230] found that the benefit of rituximab addition to hyperCVAD therapy did not extent to patients ≥60 years old, suggesting that the high-risk features (e.g., unfavorable cytogenetics) more often encountered in the older patient population cannot be overcome with rituximab. Despite an upper age limit of 59 years, Maury et al. [228] also observed that older age remained significantly associated with shorter event-free survival in the rituximab group.

Unfortunately, in all of these studies, the definition of CD20 positivity was based on the arbitrary cutoff point of 20% CD20^{POS} blasts. There is no biologic basis for using a 20% cutoff level; this custom simply refers to a time when blast cells were not gated based on CD45 intensity and other means and the 20% threshold was used to account for contaminating normal cells [231]. Second, the choice of fluorochrome used in the CD20 conjugate is essential information. It is the common experience of flow cytometrists that CD20-FITC will yield fewer CD20^{POS} blasts than CD20-PE. This is particularly obvious in chronic lymphocytic leukemia (CLL) where the leukemic B-cells express CD20 at significantly lower density than normal B-lymphocytes. As a result CD20-FITC staining on B-CLL cells is frequently too weak for accurate quantification [232]. These comments do not advise against the use of CD20 in B-lineage ALL, they merely emphasize the importance of cautiously selecting fluorochromes and knowing the consequences of the particular selection. However, in clinical trials which use a certain threshold of antigen expression to leukemic cells to trigger the use of in vivo therapy with the relevant antibody, it is imperative to declare which fluorochrome was, is, or should be used to establish that threshold of antibody binding. Ideally, a trial should be designed to give rituxan to all patients with B-lineage ALL, irrespective of their CD20 expression which should be measured using various CD20 conjugates simultaneously; subsequently, a retrospective response-driven analysis would define the prognostically significant CD20 expression level with the various CD20 fluorochromes. UKALL14 (NCT01085617), conducted by the UK NCRI Adult ALL group, is a randomized trial which adds rituximab to the treatment of B-lineage ALL irrespective of the level of CD20 expression and the correlation of response with CD20 expression will be of great interest. There is evidence that the effectiveness of rituximab correlates with the level of CD20 expression by target tissues, at least in chronic B-cell leukemias [233]. In fact, Maury et al. [228] observed a more pronounced effect of rituximab in patients with higher CD20 expression. Dworzak et al. [225] found that a cutoff value for CD20 positivity at diagnosis of 20% was insufficient to predict rituximab-induced complement cytolysis of pediatric B-lymphoblasts in vitro. These investigators also reported upregulation of CD20 expression in a significant portion of patients by glucocorticoids as early as on day 8 of therapy. Stimulation of CD20 expression has also been reported with histone deacetylase inhibitors (e.g., valproic acid), demethylating agents (e.g., azacytidine), and farnesyltransferase inhibitors [234]. On the other hand, other therapies have been found to down-modulate CD20 expression (e.g., lenalidomide, bortezomib) [234]. Rituximab binding itself leads to the depletion of CD20-expressing B-cells which, just as up- and downregulation of the molecule, occurs through a variety of mechanisms [234]. In addition to surface CD20 expression, other factors may influence the clinical benefits of rituximab treatment and contribute to antibody resistance [235]. All of these considerations further question the use of arbitrary or any cutoff points of CD20^{POS} B-lymphoblasts for defining CD20 positivity, at least with respect to the decision to add rituximab to a treatment regimen. Biologic differences between patients may also account for the inconsistent data on the prognostic significance of CD20 expression in B-lineage ALL [236–240]. Similar to early findings in pediatric B-lineage ALL [241], our analysis of the adult E2993 trial found progressively higher intensity of CD20 staining, but not the percentage of CD20-expressing B-lymphoblasts, to be associated with increasingly reduced 5-year event-free survival [Paietta E, unpublished].

Second-generation CD20 antibodies, such as obinutuzumab [242], which act through mechanisms of action different from that of rituximab, have shown activity in rituximab-resistant patients [215, 216, 227]. Of potential importance with all anti-CD20 antibodies is the occurrence of soluble CD20 antigen, since high levels of circulating soluble antigens reduce the bioavailability and thus efficacy of administered antibody [243].

CD22 Antibodies in B-ALL

In contrast to CD20, CD22 is equally expressed across all maturation stages of B-lineage ALL [244, 245]. CD22 belongs to sialic-acid-binding immunoglobulin-like lectins (siglecs), which are endocytic receptors. As a result, cytotoxic agents conjugated to a CD22-antibody are immediately internalized without shedding into the extracellular environment [245]. Another advantage is that, when compared to CD20 antibodies, CD22 expression is maintained in patients treated with CD22-targeted therapy and levels of soluble CD22 are low [245]. Thus, CD22 is an ideal target for antibody-based therapy in B-cell malignancies.

Humanized CD22 antibody, epratuzumab, has been used both unconjugated and radiolabeled form. It has a unique mechanism of action in that it modulates B-cell activation and signaling rather than eliciting direct cytotoxicity [246, 247]. Re-induction chemoimmunotherapy with epratuzumab in relapsed ALL in COG trial ADVL04P2 (NCT00098839) did not improve the clinical response when compared to historical controls, but there was a trend towards improved MRD response when epratuzumab was administered more frequently [248]. Various trials in relapsed ALL are currently ongoing (www.clinicaltrials.gov).

Inotuzumab ozogamicin (INO) is a humanized CD22 antibody attached to a toxic natural calicheamicin, a potent DNA-binding cytotoxic antibiotic via an acid-labile linker. INO has shown encouraging results in relapsed/refractory B-lineage ALL [249, 250].

CD33 Monoclonal Antibodies

CD33 is a myeloid differentiation antigen which is expressed by the majority of blasts in AML, though with variable intensities [251, 252]. Unconjugated CD33 antibodies (e.g., lintuzumab) have shown disappointing activity (rev in [252]). However, though an antigen with endocytic properties, internalization is slow, making CD33 a challenging target. Several approaches to address this problem exist [252]. Nonetheless, I will focus on gemtuzumab ozogamicin (GO) as a humanized CD33 antibody, linked to a calicheamicin derivative, given its controversial history and biologic interests. In 2000, GO was granted accelerated approval by the US Food and Drug Administration for use in relapsed AML patients ≥60 years of age. GO was voluntarily withdrawn from the US marker in 2010 due to concerns of lack of efficacy in the presence of enhanced toxicity, which emerged during an interim analysis of S0106 [NCT00085709], a SWOG-led phase 3 trial in data untreated AML [253]. While there appears to be no beneficial effect of GO on the achievement of CR, there are compelling data suggesting that GO improves survival in cytogenetically favorable (CBF leukemias) and intermediate-risk leukemias [253–255], suggesting that reapproval of GO for risk-defined subsets of AML patients, as well as APL, may be warranted [256, 257]. Various additional biologic factors affect GO efficacy, such as the presence and activity of spleen tyrosine kinase (Syk) [258], CD33 expression levels [251, 259, 260], CD33 single-nucleotide polymorphisms [261], drug efflux, toxin release, and others (rev in [262]). Increasing Syk expression, e.g., with 5-azacytidine, can indirectly enhance CD33 antibody toxicity. Along the same line, the engagement of the SHP-1 tyrosine phosphatase in Syk regulation could be pharmacologically exploited by combining CD33 antibody with cytosine arabinoside and idarubicin [222]. There are case reports on the potential efficacy of GO in CD33^{POS} ALL, but no controlled studies have been conducted.

Of note, in a recent reanalysis of trial ALFA-0701 (NCT00927498), when the level of CD33 expression by myeloblasts was correlated with the outcome with GO, the cutoff of interest was found to be 70% CD33^{POS} blasts, in that a beneficial effect of GO was only observed in this response-defined high-CD33^{+} group [260]. Once again, this data strongly supports the view that cutoff points for defining the clinically relevant levels of antigen expression in immunotherapy can only be defined after the fact when response data are available. Whether or not such defined cutoff levels will hold true for other patient populations in future trials remains to be proven.

CD52 Antibody

CD52 is expressed on virtually all lymphocytes, monocytes, and natural killer cells. In ALL, the density of CD52 antigen expression varies by lymphoid lineage, with T-lymphoblasts demonstrating significantly lower amounts of CD52 on the cell surface [110]. Data with alemtuzumab in ALL are scarce, but suggestive of antileukemic activity [263, 264]. Gorin et al. [265] tested alemtuzumab together with G-CSF (to boost antibody-dependent cytotoxicity mediated by neutrophils) in refractory and heavily pretreated relapsed ALL (NCT00773149) and saw some responses though of short duration. ECOG performed a phase 2 trial of chemotherapy combined with alemtuzumab in relapsed/refractory ALL (NCT00262925); results have not yet been published.

CD25 Antibody

CD25, the α-chain of the interleukin 2 receptor, is a powerful negative prognostic indicator both in ALL [31] and AML [32]. Until recently, the mechanisms of action of CD25 in either of these diseases have been unknown, given that CD25 does not work as part of the interleukin-2 receptor on either

lymphoblasts or myeloblasts [114]. CD25 is a transmembrane protein with a short, 13-amino acid-long cytoplasmic tail. Recent work by the Müschen group [114] has demonstrated that CD25 is a feedback regulator of the B-cell receptor (BCR) and a biomarker for disease driven by oncogenic BCR mimics. The tail of CD25 contains PKCδ substrate motifs. As a result, BCR signaling induces CD25 expression by PKCδ phosphorylation of the CD25 cytoplasmic tail which in turn leads to the membrane recruitment of phosphatases and robust oncogenic signaling (Fig. 15.2). Pharmacological activation of PKCδ, e.g., with the PKCδ agonist, diterpene ester ingenol-3-angelate (PEP005), induces rapid CD25 membrane translocation in patient-derived ALL cells. CD25 thereby mediates negative feedback signaling to stabilize oncogenic tyrosine kinase signaling and mediates drug resistance. Combinational treatment of CD25^{POS} leukemic B-lymphoblasts with PEP005- and CD25-directed immunotoxins may be a useful new approach to overcome drug resistance, for instance, in *BCR/ABL^{POS}* ALL. ADCT-301 is a human monoclonal antibody targeting CD25 linked with a pyrrolobenzodiazepine dimer toxin (CD25-ADC). CD25-ADC overcomes drug resistance in vivo when mice engrafted with CD25^{POS} human leukemic lymphoblasts are treated with this antibody conjugate. Based on this data, a clinical trial has been initiated using CD25-ADC in relapsed/refractory CD25-expressing ALL or AML (NCT02588092).

Bi-specific Antibodies

Bi-specific antibodies target leukemia-associated antigens while simultaneously activating antigens on cytotoxic effector cells or may otherwise potentiate the signaling events that will eventually lead to inhibition of leukemia cell growth. Bi-specific T-cell engagers (BiTE) form a new class of constructed antibodies, which direct the body's cytotoxic T-cells against tumor cells. One BiTE representative is blinatumomab (MT103), consisting of four immunoglobulin variable domains, of which two form the binding site for CD3 on the surface of T-cells, and the other two form the binding site for CD19 on the surface of the targeted B-cells [266]. Blinatumomab enables a patient's T-cells to recognize malignant B-lymphocytes and works by temporarily bridging these two cell types and activating the T-cells to exert cytotoxic activity against the malignant B-cells, causing redirected cell lysis, while nonspecific collateral killing effects have not been observed [267]. BiTE antibodies activate T-cells only in the presence of target cells, and nonspecific collateral killing effects have not been observed.

Blinatumomab [268] is currently indicated for *BCR/ABL^{NEG}* relapsed/refractory B-lineage ALL based on a single-arm study (NCT01209286) conducted by Topp et al. [269]. The US National Cancer Treatment Network leukemia groups are currently conducting a randomized phase 3 trial whereby patients with *BCR/ABL^{NEG}* B-ALL are randomized to blinatumomab or not at the time of complete hematologic remission based on MRD status (E1910/NCT02003222). The severe neurotoxicity seen in some blinatumomab-treated patients is not correlated with active CNS disease. Instead, variable expression of CD19 in neurons may make neurons susceptible to the inflammatory response from CD19-engaging T-cells [268]. Loss of the CD19 antigen, though rare, may contribute to resistance to blinatumomab as does extramedullary disease which probably reflects disease in sanctuary sites not penetrated by blinatumomab [268]. Duell et al. [270] recently reported that the frequency of CD4/CD25/FOXP3-expressing regulatory T-cells (Tregs), which inhibit T-cell proliferation, prior to blinatumomab administration, was able to predict blinatumomab responders. The authors also reported upregulation of CD69, CD25, and programmed death-1 (PD-1), an immune checkpoint receptor, by Tregs when incubated with blinatumomab and primary ALL blasts, suggesting a potential role of immune-inhibiting molecules, such as PD-1, in resistance to blinatumomab.

CD33/CD3-bi-specific T-cell-engaging antibody AMG 330 has shown promising ex vivo activity (rev in [271]) and has recently entered into a phase 1 trial in relapsed/refractory AML (NCT02520427). A dual-affinity-retargeting (DART) molecule generated from antibodies to CD3 and CD123 was designed to redirect T-cells against leukemic myeloblasts (also referred to as MGD006/S80880) [272]. CD123, the interleukin 3 receptor α, is highly expressed on the surface of AML blasts. Based on promising preclinical data, this antibody has entered into a phase 1 study in relapsed/refractory AML and high-risk MDS (NCT02152956).

Chimeric Antigen Receptor (CAR) T-Cells

CARs involve genetic engineering of a patient's own cells. In these molecules, an extracellular single-chain antigen recognition domain, usually derived from the variable fragment of a specific monoclonal antibody, such as CD19 (CART-19), is linked to the intracellular signaling domains of the T-cell receptor (TCR) [273]. Unlike the TCR, immunoglobulins can bind any antigen they encounter, independent of antigen processing and major histocompatibility complex. First-generation CARs, linked only to the intracellular CD3ζ signaling domain, were unable to adequately activate T-cells in vivo, and had limited clinical activity. Second-generation CARs contain costimulatory intracellular domains, such as CD28 or CD137 (4-1BB) in addition to CD3ζ [221, 274]. Third-generation CAR-T-cells which use two tandem costimulatory domains have also been reported [275]. Further improvement in efficacy and persistence of CAR-T-

cells may derive from modifying these cells to express the proinflammatory cytokine, interleukin-12, or costimulatory ligands, 4-1BB and CD40L, thus "armoring" CAR-T-cells [276]. CART-19 cells have produced promising results in various B-cell malignancies, including B-ALL [221, 277–280].

Leukemia Escape After CD19-Targeted Therapies

Fascinating data are emerging concerning the potential adaptation of leukemia cells to CD19-directed immunotherapies, both with CD19/CD3 BiTE and CART-19. Relapses with CD19^{NEG} blasts have been seen both after blinatumomab and CART-19 treatment (rev in [281]). This experience demonstrates the potent selective pressure of these therapies that drives extreme and specific escape strategies by leukemic blasts. Alternatively, anti-CD19 treatment may result in the emergence of a CD19^{NEG} clone that had been present all along as a minor undetectable subclone among predominantly CD19^{POS} blasts, following the concept of oligoclonality [282, 283]. The loss of CD19 per se has a definite impact on MRD detection in these patients. Relying on side scatter characteristics, CD45 staining intensity and CD34 expression have been suggested as a way around third problem [284]. The National Cancer Institute initiated MRD Working Group has suggested gating strategies in these patients which do not rely on CD19 alone by combining CD20, CD22, CD10, and CD24, given that CD34 is frequently not expressed in B-ALL. Of course, it is essential to make sure that myeloid cells do not contribute to the gate, given that CD24 and CD10 are expressed by normal myeloid cells and CD22 by basophils.

The diversity of mechanisms which potentially contribute to the loss of the CD19 epitope on the cell surface is astounding. Sotillo et al. described *CD19* gene mutations and appearance of *CD19* splice variants which lacked the CAR-recognizing epitope after CART-19 treatment [285]. A complete loss of antigen, rather than epitope loss or splice variant, was suggested by Braig et al. [286] who did not detect CD19 by flow cytometry using CD19 antibodies with differential epitope recognition in one case of CD19^{NEG} relapse studied in detail. In that patient, the *CD19* gene had no mutations, and full-length CD19 messenger RNA was detected, excluding transcriptional regulation and splice variants for the loss of CD19. On flow cytometric analysis, ALL blasts lacked expression of CD81 and CD21, two molecules that form the B-cell co-receptor signal transduction complex with CD19 and CD225 on the cell surface of B-cells. CD81 regulates CD19 protein maturation and acts as a chaperone of CD19 during trafficking from the Golgi apparatus to the cell surface. Rather than caused by a genetic defect, as described in antibody deficiency syndrome [287], the lack of CD81 expression in the CD19^{NEG} relapse case was attributed to posttranscriptional regulation [286]. The finding of hypoglycosylated or deglycosylated CD19 indicated that in this case of CD19^{NEG} immune escape was the result of lack of CD81 expression, resulting in defective transport and/or maturation of CD19.

To date, the other escape variation described is myeloid lineage switch [281, 288]. While the exact mechanisms of lineage switch are still to be elucidated, it is important to note that the myeloid relapses appear to be clonally related to the pretreatment disease. Gardner et al. [289] described two distinct mechanisms leading to the myeloid relapse in two cases of *MLL (KMT2A)*-rearranged ALL treated with CART-19 therapy. Secondary therapy-related AML was excluded by the identification of cytogenetic abnormalities by conventional karyotyping or FISH which were shared between lymphoid blasts before and myeloid blasts after CAR-T-cell therapy. In one case, an identical rearranged *IGH* gene sequence that was identified in both lymphoid and myeloid blasts suggested a contribution from cell reprogramming or dedifferentiation of previously committed B lymphoid blasts. However, in the second case, absence of the original *IGH* rearrangement in the myeloblasts was consistent with myeloid differentiation of a non-committed stem cell clone or selection of a preexisting myeloid clone. These authors discussed as another alternative the outgrowth of a CD19^{NEG} myeloid clone in response to cytokines. Both patients with phenotypic switch had presented with severe cytokine release syndrome (CRS), including high serum levels of interleukin-6, a well-known complication of CAR-T-cell therapy [220, 221, 290, 291]. Other patients in the same treatment cohort, who did not experience phenotype switch, did not experience severe CRS [289]. In vitro IL-6 supplementation has been found to be a key factor in driving myeloid differentiation of a t(4;11) *MLL(KMT2A)*-rearranged B-ALL line [292], suggesting that high serum cytokine levels during CRS might contribute to the myeloid switch phenomenon encountered after CART-19 therapy. On the other hand, a clonal phenotypic switch was also described in a t(4;11) ALL patient following blinatumomab without any obvious sign of cytokine contribution [293].

How to deal with CD19 loss therapeutically or how to avoid it is a work in progress [221, 274, 281] and beyond the scope of this chapter.

Minimal Residual Disease Determination by Flow Cytometry

The topic of MRD in leukemia is comprised of several aspects, including (1) the most advantageous methodology for MRD assessment, (2) the selection of peripheral blood

versus bone marrow and the issue of sample quality, (3) the timing of MRD assessments, (4) the clinically relevant level of MRD, and (5) the association of flow cytometry with other prognostic factors, in particular, genetic aberrations. This section of the chapter focuses on the flow cytometric detection of MRD and biologic parameters associated with or predictive of MRD.

MRD by definition refers to persistent disease after treatment that remains at levels too low to be detected by the human eye with light microscopy. MRD reflects the quality of response, serves as one of few post-therapy prognosticators, and has been found to prognostically significant at all time points though at different thresholds; furthermore, MRD thresholds will change with novel therapies and must be established with the introduction of new treatment strategies. The optimal way for detecting MRD has yet to be established, particularly in AML. Despite a lack of assay standardizations and the use of variable thresholds for defining MRD positivity, the prognostic effect of MRD has been confirmed universally indicating that this parameter is not easily swayed by technological aspects. There exist standard pre-therapeutic prognostic features, including cytogenetic aberrations, gene mutations, and immunophenotypic characteristics, which predict for the occurrence of MRD after therapy. However, within each conventional risk category, MRD status adds independent prognostic information. For example, patients with favorable cytogenetics will do much more poorly if they remain MRD^{POS} after therapy than favorable-risk patients who become MRD^{NEG}. On the other hand, intermediate-risk patients who become MRD^{NEG} with induction chemotherapy may do as well as MRD^{NEG} favorable-risk patients. In theory, early MRD assessment in the treatment course allows for refined risk stratification and tailored treatment. There are numerous reviews on MRD of which only a few recent ones are listed here, a list which by no means claims to be inclusive [34, 294–303].

New Definition of Complete Remission

It has almost become the standard of care to determine MRD in patients who after induction chemotherapy achieve a morphologic complete remission (CR). The implication that MRD might exist at the time of morphologic CR was raised by Bradstock et al. in 1981 [304] in patients with T-ALL, using the limited antibodies available at that time. It is quite unbelievable that despite 35 years of increasing evidence in favor of a prognostic significance of MRD in morphologic remitters, most clinical trials still define CR as the absence of blasts in the blood, normal trilineage hematopoiesis, ≤5% blasts in the marrow as recognized by the human eye with light microscopy, and absence of extramedullary disease [305]. We will discuss some of the reasons for this lag in reaction time, most of which appear to stem from lack of reliability in MRD measurements. Current CR subcategories, CR with incomplete blood count recovery (CRi), and its subset, CR with incomplete platelet recovery (CRp) [305], are associated with inferior long-term survival than patients in morphologic CR [306]. Chen et al. [307] found that MRD levels in marrows from CRi patients are significantly higher than in those from morphologic CR or CRp patients. Freeman et al. [308] had previously suggested a high incidence of MRD positivity in CRp patients and the loss of prognostic power for insufficient platelet count recovery in multivariate analysis after adjustment for MRD status. A major step in the direction of MRD has been taken by the European LeukemiaNet recommendations 2017 for the diagnosis and management of AML in adults [309] by introducing new response criteria, CR without MRD and the conventional morphologic CR with unknown or positive MRD. They also recommended that both multiparameter flow cytometry (MFC) and molecular techniques can be used to monitor MRD in AML. These recommendations will hopefully impact response criteria in clinical trials and individual patient treatment plans.

Of great importance are results from COG trial AAML03P1 [NCT0007017], which suggest that about 30% of children with AML who have a morphologically positive marrow after induction and are, therefore, considered induction failures are negative for MRD by MFC [310]. These children have an outcome superimposable to that of patients who were negative for MRD by both methods. Inaba et al. [311] similarly reported some AML patients who by morphologic examination of the bone marrow after induction had >5% blasts, while MFC-MRD did not detect residual leukemia. Percentage of myeloblasts by morphology did not affect the relation between MFC-MRD and treatment outcome marrows. These authors hypothesized that MFC-MRD negativity in morphologically positive marrows was due to therapy-induced differentiation of leukemic cells, resulting in the observed discordance between antigen expression and morphologic appearance. Since maturing blasts are doomed to undergo apoptosis, such patients would be expected to have a favorable outcome indistinguishable from that of MRD^{NEG} patients. In APL patients treated with all-*trans* retinoic acid, early MRD assessment by PCR for the *PML/RARα* transcript is not informative because the transcript persists in leukemic promyelocytes which undergo retinoic acid-induced maturation [312]. Such complication of MRD measurements does not occur in APL treated with arsenic trioxide [313]. However, a similar interference with MRD detection can be expected from other differentiation-inducing therapies, such as with inhibitors targeting *IDH2*-mutant AML [314]. Using real-time quantitative (RQ-PCR) for somatic receptor gene rearrangements, O'Connor et al. [315] analyzed end-of-induction MRD in pediatric ALL from

MRC trial, UKALL2003 [NCT00222612]. Of >3000 patients, only 1.9% were morphologic induction failures, and of those 59 patients, 6 had between 5 and 25% blasts by morphology (M2 marrow) but <0.01% MRD by PCR. These MRD$^{\text{VERY LOW}}$ patients had a 5-year event-free survival of 100%. These findings are surprising. What is surprising is the fact that despite the known limitations of morphology, these studies have not been done earlier and are still not being done in major trials. Going forward, every patient with AML and ALL, whether an induction failure by morphology or a morphologic remitter, must be evaluated for MRD by more sensitive methods to confirm or negate the morphologic result. Only then can we make sure that patients are treated according to their true depth of induction response.

The Choice Between Bone Marrow and Blood for MRD Detection

MRD determination by MFC invariably finds a lower percentage of blasts in bone marrow aspirates than in blood, provided that the aspirate is not diluted with blood. While this statement clearly applies to B-ALL, there may not exist such difference between marrow and blood in T-ALL [316]. This is in agreement with the fact that B-ALL (and AML, see below) are malignancies of the bone marrow, while T-ALL is of thymic origin. We have found in a large fraction of adult B-ALL patients that MRD by MFC was undetectable in the blood when up to 5% of blasts were measured in the marrow [317] and this experience continues in ongoing trial, E1910 [NCT02003222] (Paietta E unpublished observation). Along the same line, van der Velden et al. [318] found that RQ-PCR monitoring of immunoglobulin and T-cell receptor rearrangements yielded up to 1000 times higher levels of MRD in the bone marrow of children with B-ALL compared to levels in the blood. On the other hand, levels in the two tissues were comparable in T-ALL patients. Based on the available literature, MRD levels in the blood are 1–3 logs lower than in bone marrow in B-ALL and comparable or 1 log lower in T-ALL. Consequently, for both leukemia subtypes, bone marrow sampling was pronounced a prerequisite for accurate MRD monitoring (rev in [294, 297]).

Remarkably, a recent review stated that studies in ALL have already proven that assessment of bone marrow MRD could be replaced by peripheral blood MRD, without giving a reference or eluding further on the subject [296]. As stated above, this is not the case. However, there are some additional interesting data on this issue. Coustan-Smith et al. [316] reported from a small number of pediatric B-ALL that those patients with detectable MRD in both blood and marrow at the end of induction had a significantly higher incidence of relapse than patients with MRD in bone marrow only. It is important to mention here that their flow cytometric assay at the time only detected 1 leukemic cell in 10,000 normal cells, a sensitivity 10 times lower than what we are used to seeing now. Despite this limitation, their results suggested that peripheral blood MRD in B-ALL, though significantly lower than in the marrow, could provide prognostic information. An analysis of >2000 children with B-lineage ALL by Borowitz et al. [319] revealed that detection of bone marrow MRD at the end of induction helped to identify patients at high risk of relapse, but was not as useful for identifying patients at low risk. In multivariate analysis, MRD positivity (>0.01%) by MFC on day 29 (end of induction) was the most significant prognostic factor across all risk groups. While peripheral blood MRD was not equivalent to bone marrow MRD, the presence of MRD on day 8 in peripheral blood was associated with adverse outcome, with an increasingly bad outcome seen with progressively higher MRD levels. Strikingly, day 8 blood MRD$^{\text{NEG}}$ patients had a better prognosis than patients who were bone marrow MRD$^{\text{NEG}}$ on day 29. And the group of patients with the best outcome were MRD$^{\text{NEG}}$ in blood on day 8 and had favorable cytogenetics. This prognostic significance of early blood MRD was also confirmed in a small study by Volejnikova et al. [320] using RQ-PCR for somatic receptor gene rearrangements. Once again, these authors confirmed a poor correlation between MRD levels in marrow and blood in B-ALL. However, they also found that a negative MRD result in the blood on day 15 identified a group of patients with excellent relapse-free survival. In summary, MRD levels in the blood are not equivalent to those in the marrow. Blood MRD status early on during induction chemotherapy in B-ALL, however, carries prognostic significance.

Measuring blood MRD on day 8 with MFC presents with a major challenge in that WBC counts will be markedly suppressed by chemotherapy. Rarely will it be possible to acquire the desired number of events (500,000–1 million), thus reducing sensitivity of MRD detection [319]. One clear advantage of blood when compared to bone marrow for MRD detection in B-ALL is the absence of normal B-cell precursor cells (hematogones) which occur in increased numbers in the marrow especially after consolidation therapy as part of marrow regeneration and which can be mistaken as persistent MRD [35, 297, 321]. In other words, the sensitivity of the blood MRD assay is lower compared to that of marrow MRD determination, while blood MRD has higher specificity due to the relative absence of normal progenitors in the blood. Next-generation sequencing (NGS)

in paired bone marrow and blood samples from patients with B-lineage ALL also found that the leukemia burden in the blood was sixfold lower than in marrow [323]. However, due to the extremely high sensitivity of NGS, only 17% of paired samples had disease detectable in marrow but not peripheral blood and those were predominantly cases with marrow MRD ≤0.01% of total cells. These data suggest that with NGS, blood might be an alternative source tissue to marrow for MRD detection.

Regarding AML MRD, comparative data in blood and marrow are scarce. In 2007, Maurillo et al. [324] reported a strong concordance between MRD levels in blood and marrow by MFC and determined that a blood MRD level of 0.015% post-induction or post-consolidation correlated with outcome. Of the 50 patients studied, 43 had blood MRD levels higher than 0.015% after induction and 77% of those relapsed after a median of 10 months. Regarding biologic characteristics of this patient cohort, there was an overrepresentation of intermediate-risk karyotypes and cytogenetics had no effect on outcome. It is noteworthy that seven patients with detectable blood MRD after consolidation have remained in continuous remission, questioning whether the cutoff of 0.015% may be clinically relevant. There was no difference in overall survival between patients positive for blood MRD after induction and those with MRD >0.015%, though relapse-free survival was significantly shorter in the MRD^{POS} group. The same group of investigators use a threshold of 0.035% to define MRD positivity in the marrow [325]. Maurillo et al. [324] mentioned three patients in whom MRD levels in the blood were tenfold higher than in the marrow and these patients were monoblastic leukemias with extramedullary disease at the time of study. Zeijlmaker et al. [326] also reported one case with FAB M5 leukemia in whom MRD levels in the blood were 6.9 times higher than in the marrow, though extramedullary disease was not present. These investigators analyzed paired marrow and blood samples from 114 AML patients and found blood MRD levels to be 4–5 times lower in the blood based on MFC. Blood MRD was an independent predictor of response duration using a threshold of post-induction and post-consolidation MRD of 0.04% to distinguish between MRD^{NEG} and MRD^{POS} status, which was based on optimal specificity. This threshold agreed with the median of primitive marker-positive cells (CD34^{POS}, CD117^{POS}, or CD133^{POS}) in the blood of healthy individuals (0.038%) as compared to a median percentage of 0.88% in the marrow of these controls. Although the frequency of the primitive cell population itself was lower in the blood of AML patients, expression of aberrant markers by the primitive cells was not. With regard to the use of blood as an alternative source to marrow for MRD detection, they made the reasonable suggestion to monitor MRD in the blood and to assume that a patient is at high risk for relapse if the blood is MRD^{POS}. In cases in which the blood is MRD^{NEG}, an additional marrow aspiration should be performed to evaluate the true MRD status.

The Sample Quality Conundrum in MRD Determination

I cannot emphasize enough that it is the quality of the aspirate that determines the accuracy of MRD results. Several factors affect bone marrow quality and can be detrimental to accurate MRD determination. Many of those are out of the hands of the laboratory eventually performing MRD analysis. Those pre-analytical factors are sample storage after collection, delay in transport to the laboratory, or damaging conditions during transport (e.g., heat), resulting in degenerative changes with preferential loss of cell populations, possibly including the blasts in question. Hemodiluton of bone marrow samples, however, is probably the most significant and unfortunately too common problem. In an elegant, though mostly overlooked study, Helgestad et al. [327] demonstrated that the technique of bone marrow aspiration dramatically influenced MRD levels in ALL. Even the second pull from the same aspiration site reduced the leukemic fraction in the aspirate by almost 50% due to dilution with blood containing much fewer blasts. As a result, clinical trials in pediatric as well as adult B-ALL now emphasize the need of a first pull aspirate for MRD evaluation, or a separate pull from a distinct aspiration site after redirecting the needle, to avoid hemodilution at all costs as it leads to underestimation of leukemia involvement. Loken et al. [328] suggested a method to normalize aspirates for hemodilution flow cytometrically based on the proportion of dim-staining CD16^{POS} maturing myeloid cells, which, however, has not found widespread application. An intriguing suggestion as to how to resolve the problem of hemodilution in MRD detection was made by Terwijn et al. [329]. These investigators related the fraction of malignant primitive cells, those expressing aberrant markers (e.g., CD7) (aPC), to the total population of primitive cells, based on the dim expression of CD45, low side scatter characteristics, and expression of primitive markers, CD34, CD117, or CD133. In their experience, in contrast to MRD, the aPC fraction did not decrease upon dilution of the marrow with blood, because aPC fractions in peripheral blood and bone marrow were comparable.

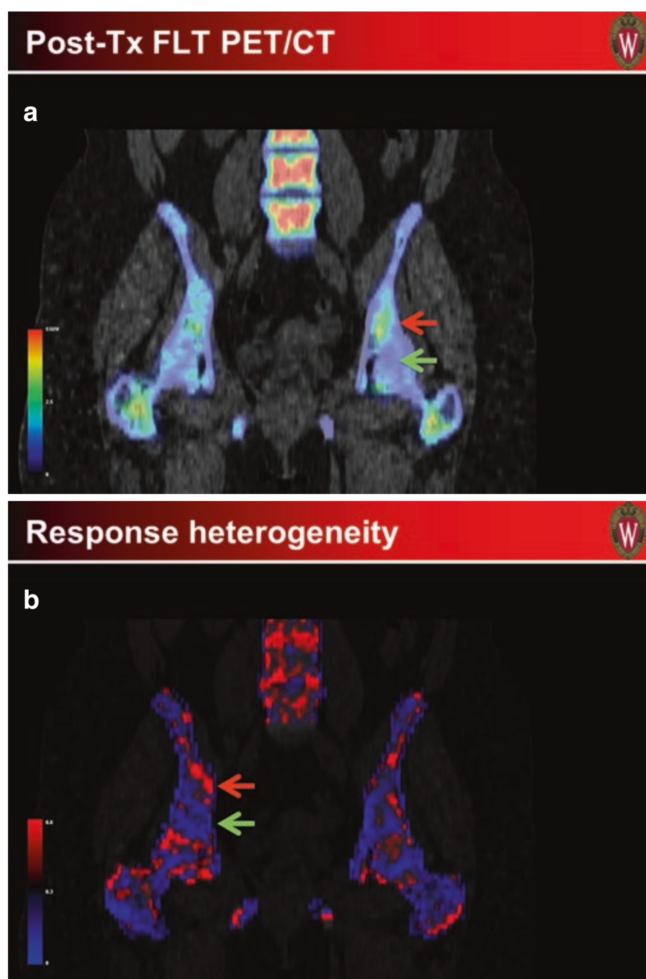

Fig. 15.4 FLT PET/CT images of posttreatment bone marrow in an AML patient. The patient was injected intravenously with 5 mCi FLT and scanned using a GE Discovery LS PET/CT scanner at the University of Wisconsin. PET/CT imaging began 45 min post-injection and extended inferiorly from the top of the skull to the distal femora. The pelvic region was chosen for display in this figure given that the iliac cristae are the most commonly used site for bone marrow aspiration and biopsy. PET activity concentrations were converted to standardized uptake values (SUV) by dividing the activity concentrations by the injected activity per patient mass. FLT PET images of bone marrow proliferative activity were extracted from full FLT PET/CT scans by first using the CT scans to extract the bone. These CT bone volumes were expanded to include the marrow resulting in bone and marrow CT masks. These masks were applied to the FLT PET images to isolate FLT PET voxels (volume elements of a scan) representing proliferative activity of bone marrow. An SUV threshold of 0.5 was applied to the masked FLT PET images to extract marrow FLT PET voxels, yielding FLT PET images of bone marrow proliferation. (**a**) Shows the FLT PET/CT of bone marrow in the pelvis and lower lumbar spine in an AML patient post-induction therapy. The SUV color bar reflects high proliferative activity in red (*red arrow* and red areas in the vertebrae) and low proliferative activity in blue (*blue arrow*). This scan demonstrates the heterogeneity of proliferative activity and thus distribution of residual disease in the pelvic regions. (**b**) Shows the spatial heterogeneity of bone marrow response to therapy in the same AML patient. The image represents a voxel-by-voxel ratio, whereby the values in the posttreatment FLT PET/CT were divided by the values in the pretreatment FLT PET/CT after co-registration of the pre- and posttreatment scans. Regions in red contained about 60% of the pretreatment activity, regions in black contained about 30%, and those in blue represent complete response. As can be seen from both images, there was substantial heterogeneity of residual bone marrow, as well as bone marrow response throughout the pelvic region, emphasizing the issues with a single-site bone marrow biopsy to assess treatment efficacy and response (courtesy Dr. Robert Jeraj)

Differential, heterogeneous distribution of the tumor load in different parts of the bone marrow, especially after treatment, could result in erroneously low MRD levels. In ALL, two independent bone marrow punctures at different locations showed comparable levels of MRD [330] strongly suggesting an equal distribution of ALL cells and indicating that it is sufficient to analyze MRD in a single bone marrow sample only. Substantial spatial heterogeneity in the bone marrow response after treatment for AML has been revealed by positron emission tomography (PET) with the proliferation marker, 3′-deoxy-3′-[^{18}F]fluoro-l-thymidine (FLT), in that response varied considerably throughout the pelvic region and in relation to other parts of the bone marrow [331]. Both during and after therapy, FLT uptake in the bone marrow of patients with residual disease, as assessed by bone marrow biopsy on day 14, was much higher and much more heterogeneous than in patients who achieved a morphologic CR. Figure 15.4 demonstrates the heterogeneous distribution of non-proliferating and actively proliferating bone marrow in the pelvis of an AML patient after induction chemotherapy (Fig. 15.4a). Figure 15.4b shows considerable spatial heterogeneity in the bone marrow response of an AML patient with refractory disease visualized by comparing pre- and posttreatment proliferative activity (disease) throughout the pelvis. Interestingly, there was a suggestion that elevated pretreatment FLT uptake may be predictive of poor response to induction, in agreement with data from Buck et al. who observed the highest FLT uptake in bone marrow of patients with refractory leukemia [332]. In other words, the accepted practice of performing single-point measurements for MRD in the bone marrow of treated AML patients could yield a false-negative result due to the heterogeneity in disease distribution. A similar study by Weindel Ibar Cribe et al. [333] also found a high degree of consistence between bone marrow response in AML patients to induction chemotherapy and the results of ^{18}F-fluorodeoxyglucose PET scans. These investigators speculated that a suboptimal PET response may be a predictor for an early relapse. The ECOG-ACRIN Cancer Research Group (Eastern Cooperative Oncology Group [ECOG], American College of Radiology Imaging Network [ACRIN]) is currently performing a trial to formally assess the value of FLT imaging in comparison to flow cytometric and morphologic MRD assessment in the marrow on day 28 of induction (EAI141, registered as NCT02392429).

A packed or concentrated marrow can be fully replaced by malignant cells at 100% cellularity leading to dry taps or the inability to obtain a suitable aspirate with spicules. Another etiology of dry taps can be reticulum fibrosis. Bone marrow fibrosis at diagnosis, as measured by reticulin fiber density, has been found to be more common in B- than T-ALL and has been associated with higher MRD levels on day 29 in B-ALL [334]. Packed marrows are much more common at diagnosis than after treatment and thus have less impact on MRD detection than hemodilution due to incorrect marrow aspiration.

MFC-MRD Versus Molecular MRD

The detection of MRD by MFC is based on the principle that leukemic cells express aberrant patterns of antigens which allow us to distinguish them from normal cells of same lineage and similar maturation stage [335–339]. The optimal method of MRD quantification and the best way to incorporate MRD information into risk-adapted strategies remain to be determined. The big advantage of MFC-based MRD assessment is its broad applicability. Leukemic blasts from approximately 90–95% of acute leukemia patients present with one or more antigen profiles which differ from the ordered pattern of antigen expression usually seen in normal hematopoiesis.

Flow cytometry looks at viable cells, which is an advantage over molecular techniques that may detect residual disease stemming from apoptotic or partially differentiated blast cells which have lost both aberrant phenotype and clonogenic potential. Inaba et al. [311] demonstrated this phenomenon when they compared MRD detection by MFC and qualitative PCR in CBF leukemias in which MRD by MFC after induction improved risk assessment, whereas the mere detection of the relevant transcripts by PCR did not. Low levels of molecular MRD may be compatible with long-term survival in CBF leukemias, again suggesting persistence of fusion transcripts in pre-leukemic cells or in differentiating leukemic cells without leukemogenic potential [340]. Complementary roles of MFC and RQ-PCR for the monitoring of MRD in CBF leukemias have been recently suggested [341].

With continuous improvements in the MFC methodology for MRD detection, differences in sensitivities between MFC and molecular techniques are diminishing. The standardized eight-color MFC-MRD procedure developed by the EuroFlow Consortium has proven a 98% concordance between MFC and RQ-PCR of immunoglobulin/TCR rearrangements in B-ALL [303], whereby the increased sensitivity of MFC was due to the availability of <4 million cells per antibody tube. Initially discordant cases were either due to overinterpretation of MFC-MRD (calling actually MRD^{NEG} cases MRD^{POS}) which were resolved by reanalysis or due to nonspecific amplification leading to false-positive RQ-PCR data. In those false-positive RQ-PCR cases, qualitative NGS analysis could not detect the involved leukemia-specific immunoglobulin/TCR rearrangements. The EuroFlow Consortium has reached extreme standardization of pre-analytical and MRD procedures (www.EuroFlow.org). Their EuroFlow quality assurance program helps to identify technical failures and inconsistencies [342]. Up until now, they have recommended using the Infinicyt software for analysis, which compares leukemic B-lymphoblasts with the nearest-normal B-cell precursors using automated population separator plots. Still, Theunissen et al. [303] concluded that interpretation of MFC-MRD, especially at very low levels, remains expert based. Being true to the term EuroFlow-based next-generation flow (NGF)-MRD strategy [294], EuroFlow now strives at developing new software tools to reduce subjectivity in MRD interpretation. The main limitation for MFC-based MRD evaluation, in fact, is the need for data interpretation which, in the hands of the untrained, is as subjective as the morphologic assessment of bone marrow smears. In other words, detailed knowledge of normal maturation for cells of the relevant lineage is an absolute prerequisite for the correct recognition of MRD by MFC [343, 344]. A recent first effort by the National Cancer Institute to standardize MFC-MRD for B-ALL in North America, using the COG-protocol for B-ALL as the gold standard, has demonstrated that a standardized methodology and continuous educational feedback helped improving the performance of participating laboratories [345]. While the COG protocol is based on the different-from-normal (DFN) approach, similar experience was reported by Feller et al. [346] who aimed at concordance of data in AML among five centers utilizing leukemia-associated immunophenotype (LAIP)-based MFC (DFN versus LAIP concepts of MFC-MRD determination will be discussed below). In both attempts, discordances were interpreter dependent, as previously observed in earlier European standardization efforts for B-ALL MRD assessments in multicenter settings [347, 348]. Since participants in all of these trainings were experienced in standard diagnostic flow cytometry of leukemias, these results suggest that immunophenotypic MRD assessment requires additional guided schooling.

Molecular studies are limited by the requirement of predefined targets, such as leukemia-specific, recurrent translocations leading to the creation of fusion transcripts (e.g., *BCR/ABL*), somatic gene mutations (e.g., *NPM1, FLT3, DNMT3A*), or, in ALL, creation of patient-specific primers for immunoglobulin and/or TCR gene rearrangements unique to the abnormal clone present at diagnosis. Good correlation has been found between RQ-PCR amplification of most frequent fusion transcripts in B-ALL and MFC-MRD [349]. Discordance between RQ-PCR MRD and MFC-MRD in *ETV6/RUNX1* (*TEL/AML1*) ALL affected only early time points of induction chemotherapy. It is possible that RQ-PCR detected *ETV6/RUNX1* carrying pre-leukemic clones (CD34^{POS}CD38^{NEG}CD19^{POS} pro-B-cells), which by MFC were not recognized as leukemic B-lymphoblasts [350]. Molecular signals may have derived from fusion transcripts present in nonviable or differentiating blast cells [340]. Huang et al. [349] speculated that the sensitivity of their six-color MFC-MRD assay could have been increased by adding additional antibodies to newly described markers to their panel [351]. Eight-color flow cytometry has yielded improved concordance with molecular MRD in BCR/ABL^{POS} ALL [352].

Ravandi et al. [353] reported that >50% of MRD specimens from their *BCR/ABL*^{POS} ALL patients were *BCR/ABL*^{POS} by PCR but negative by MFC. These findings are in agreement with those of others who found that MRD levels by MFC and/or PCR amplification of antigen receptor genes are largely equivalent but different from *BCR/ABL* transcript quantification [354]. It is conceivable that multi-lineage involvement with the presence of *BCR/ABL* in non-ALL

cells is in part responsible for this finding [355]. More recently, the suppression of BCR/ABL protein translation in LSCs which carry *BCR/ABL* transcripts has been reported in imatinib-refractory chronic myeloid leukemia, resulting from the modulation of LSC metabolism by the environment in the stem cell niche [356]. The detection of MRD which is represented by residual LSCs is essential for long-term outcome in *BCR/ABL*^{POS} disease. A potential marker of *BCR/ABL*^{POS} LSC, dipeptidyl peptidase IV (CD26), has been proposed as a follow-up parameter at least in TKI-treated CML and lymphoid blast crisis of CML [357].

Correlations between MFC-MRD and molecular MRD in ALL have mainly focused on immunoglobulin/TCR gene rearrangements [340, 351, 358–360]. The two strategies were found to be complementary and (almost) comparable when properly standardized and the choice between these two methods is primarily dictated by costs, time, and expertise available. While fusion transcripts represent very stable targets for MRD determination, clonal evolution of immunoglobulin and TCR gene rearrangements is a frequent occurrence with therapy [361]. NGS, but not allele-specific oligonucleotide (ASO)-PCR, allows for the tracking of the evolution of all gene rearrangements during therapy [362–364]. Not surprisingly, deep sequencing by NGS measuring immunoglobulin heavy-chain variable, diversity, and joining DNA sequences proved superior to six-color MFC-MRD when tested prior to and early post-allogeneic stem cell transplantation [365]. With a sensitivity level 1000-fold lower than that of optimal MFC-MRD, pre-transplant NGS-MRD negativity, but not MFC-MRD negativity, was strongly correlated with relapse-free survival, raising the question whether the potential toxicities of allogeneic transplantation could be avoided in this patient cohort.

With respect to common gene mutations in AML, PCR- and sequencing-based MRD monitoring confirmed heterogeneity and clonal instability between diagnosis and relapse with selective outgrowth of mutated clones (oligoclonality), in particular, for *FLT3-ITD* [366], while *NPM1, DNMT3A*, and *IDH2* mutations were found to be more stable [282, 367–369]. Salipante et al. [369] reported *NPM1*-mutation-positive MRD by NGS in six out of six randomly selected AML patients after therapy though they were MFC-MRD negative, possibly reflecting persistence of mutated *NPM1* in a pre-leukemic clone, carrying also mutations in epigenetic landscaping genes (e.g., *DNMT3A, IDH1/IDH2*) [22]. Ivey et al. [370] found that in patients in molecular remission according to the *NPM1* mutation RQ-PCR assay, the coexisting *DNMT3A* mutations at arginine 882 (*DNMT3A*^{R882}) persisted, suggesting that they drive relapse [21]. The presence of *DNMT3A*^{R882} mutations predicted MRD measured by six-color MFC in AML patients in first remission, treated on phase 3 trial, E1900 [NCT00049517], underscoring their role in chemoresistance [148]. In that study, none of the other gene mutations analyzed, including mutated *NPM1* and *FLT3-ITD*, was associated with MFC-based MRD.

The relationship between *BCR/ABL1*-like genotype and MFC-MRD was studied by Roberts et al. in the St. Jude Total Therapy XV trial [204]. In that study, the majority of patients with *BCR/ABL1*-like ALL had an inferior initial response to treatment which triggered them being treated with intensified therapy. This MRD-guided risk assessment yielded comparable clinical results in *BCR/ABL1*-like and other B-ALL. Based on these findings, the authors proposed for centers that lack the capability to identify *BCR/ABL1*-like ALL that excellent overall treatment results can still be achieved, provided that reliable methods for monitoring MRD are available.

How to Define MRD Positivity and Negativity

In the study by Pulsipher et al. [365], ALL patients with even the lowest levels of residual disease by NGS (10^{-6} to 10^{-7}) benefitted from the allotransplant as patients with such low MRD levels who did not get a graft-versus-leukemia effect from the transplant relapsed. A strict correlation between the presence of quantifiable MRD pre-transplantation and outcome in ALL has also been reported by others [181, 371, 372], whereas this absolute need for MRD reduction may [373] or may not apply to AML [371, 374]. The power of pre-allotransplant MRD in AML has been demonstrated by a meta-analysis which demonstrated a strong relationship between pre-transplant MRD and post-transplant relapse and survival which was independent of the methodology used to determine MRD, the MRD threshold, as well as the conditioning regimen used [375].

MFC-MRD levels (threshold 0.01%) correlated with the outcome of myeloablative allogeneic stem cell transplantation in pediatric ALL with 3-year estimates of relapse being 17% and 38% and estimated 3-year overall survival being 68% and 40% for patients without and with MRD prior to transplant, respectively [376]. While these differences were highly statistically significant, they indicated that 17% of patients with <0.01% MFC-MRD relapsed within 3 years after transplant. This observation suggests that prognostic heterogeneity exists within the MRD^{NEG} group when defined by the 0.01% threshold. That the commonly used threshold of 0.01% to define MRD positivity in ALL, while usually achievable by MFC, may be set too high for clinical relevance in all ALL patients has also been suggested by PCR amplification of antigen-receptor genes at the end of remission induction [377]. On the other hand, in the first clinical trial in pediatric ALL that used MRD levels prospectively during and after remission induction therapy to guide risk-directed treatment, the St. Jude Total Therapy XV study (NCT00137111), the threshold for determining 10-year event-free survival was

≥1% of MFC-MRD on day 19 of induction [378]. However, at later time points of therapy, including prior to allotransplant, MRD^{NEG} status was defined with <0.01%. As pointed out by Campana and Coustan-Smith [340], the proportion of MRD^{POS} samples at any given time point during the course of treatment is highly dependent on the preceding therapy. Using a threshold of ≥0.01% to define MRD positivity, these authors stated that the prevalence of MRD positivity at the end of remission induction for ALL ranged from 19.4 to 83.5% in a variety of studies. This implicates that clinically meaningful MRD levels need to be determined with every new therapy. There is evidence that end-of-induction MFC-MRD is not as useful a determinant of outcome in T-lineage as in B-lineage ALL [379], consistent with PCR data which showed that T-ALL patients with MRD at the end of induction and even end of consolidation had a favorable outcome [380].

For MFC-based MRD in AML, the cut point used to define MRD positivity is, in general, ten times higher than in ALL (≥0.1%) [325, 381, 382]. To determine the cutoff of MRD positivity most relevant for relapse-free survival early during induction therapy, at the time of aplasia, Köhnke et al. [383] calculated hazard ratios for different cutoff values from 0.01 to 1% and arrived at 0.15% patients with ≥0.15% of mononuclear cells exhibiting the leukemia phenotypes(s) that were considered MRD^{POS}. Utilizing the same method, a cutoff of 0.3% was determined as to represent MRD positivity post-induction. Lowering the cutoff to 0.1% at that time point resulted in a nonsignificant correlation with survival.

In the pediatric AML02 trial (NCT00136084) [384], a rare example of MRD-guided therapy in AML, an MRD level of ≥1% after the first course of induction therapy was the determining factor for outcome in these high-risk patients, despite treatment intensification triggered by MRD results, suggesting that novel therapies might be indicated for this cohort. Patients with low MRD levels after induction 1 (0.1 to <1%) did as well as MRD^{NEG} patients, whereas any detectable MRD (>0.1%) after induction 2 was associated with poor outcome. Buccisano's group [385, 386] suggested that the clinically relevant threshold of MRD will differ with the intensity, in other words, the efficacy, of the therapies administered. In their experience, the MRD level that discriminated MRD^{NEG} from MRD^{POS} AML patients decreased from 0.1 to 0.035% in the bone marrow after intensification of the therapeutic schedule. On the other hand, high-dose versus low-dose cytarabine during induction did not affect post-induction MRD levels in pediatric AML, though this study demonstrated an MRD-lowering effect of gemtuzumab ozogamicin, the anti-CD33 antibody, when given after the first course of induction [384], and neither did doubling of the daunorubicin dose in adults [387], although the latter resulted in prolonged overall survival [388]. Similarly, there was a significantly better outcome in relapsed ALL children induced with mitoxantrone versus idarubicin without apparent difference in post-induction MRD levels [389]. Butturini et al. [390], however, warned about applying a definition of MRD status which was established in a given clinical situation in a random fashion to other settings. In other words, MRD thresholds that are prognostically significant in children with de novo ALL treated with standard therapy may not be applicable to children in relapse treated with experimental regimens. Yang et al. [391] identified germline variations which distinguished children at risk of relapse despite an excellent early response to therapy. Of the 134 single-nucleotide polymorphisms associated with relapse, 14 were associated with unfavorable pharmacokinetics of commonly used antileukemic drugs.

Intensification of induction chemotherapy may indeed lower the threshold to be used for defining MRD negativity to undetectable, given that patients who remain with detectable disease after highly cytotoxic therapy must be considered as having a low likelihood of benefitting from further cytotoxic therapy, most likely including transplantation [392]. Araki et al. [373] demonstrated that AML patients in MFC-MRD^{NEG} remission prior to myeloablative allogeneic transplantation had a cumulative risk of relapse of only 20–25% compared with a relapse risk of 65–70% for patients who underwent transplantation while in MRD^{POS} remission or patients with morphologically visible disease. Importantly, MRD negativity was defined as the absence of detectable MRD with ten-color MFC and the different-from-normal (DFN) MRD assay approach. Outcomes in patients with MFC-MRD^{POS} were unrelated to the level of MRD (<0.1% to >1%). The reason why about one-quarter of remitters relapsed posttransplant despite having been MRD^{NEG} prior to the procedure might at least in part be explained by the threshold of 0.1% to determine MRD negativity which falsely identified these patients as free of MRD.

Given the complex heterogeneity of AML, in addition to methodological disparities in MRD assays, it is probably reasonable to propose that the absence of detectable MRD, at any time point of treatment, should determine MRD negativity [393]. One of the main clinical uses of MRD determination is monitoring the kinetics of MRD reduction with treatment, rather than a single-time-point assessment. The usefulness of detecting recurring MRD in patients in continuous hematologic remission will depend on the availability of MRD-targeting preemptive therapeutic strategies [381].

Sample Preparation for MFC-MRD Determination

If MRD negativity is to be determined by the inability to detect MRD, then the lowest level of detection will dictate this definition. Consequently, unless everybody utilizes the same methodology for MFC-MRD assessment, it will continue to be impossible to compare studies. In addition to the

caveat of threshold determination for MRD status and the choice of MRD assay, as discussed below, there are differences in sample preparation which can markedly affect results: for example, MRD determination in mononuclear cells versus whole bone marrow. While MFC-MRD data from mononuclear cells may be better comparable to those from molecular MRD analysis (nucleic acids are always prepared from mononuclear cells), there is the possibility that MRD cells are lost during density gradient centrifugation and/or antigen expression is altered during the procedure. Furthermore, Gaipa et al. [394] found that the impact of using either monucleated or total nucleated cells on the concordance of MFC with PCR results was minimal, and rather influenced by the time point at which MRD was measured. While direct antibody staining of MRD cells in whole, unseparated tissues is the preferred method nowadays, a no-wash procedure [395] is ill advised. It is imperative that excess antibodies be washed away after incubation and before red cell lysis and fixation prior to acquisition [297].

Optimally, a cluster of ten clearly aberrant cells can be sufficient for the recognition of MRD in a well-controlled assay. In that setting, for a limit of detection of 0.01%, the threshold currently utilized in B-lineage ALL to define MRD status, 1 million events must be acquired. Notably, in the presence of platelets, red cell fragments, tissue debris, or nonnucleated cells more events must be acquired, unless this noise can be excluded from the MRD enumeration. Other factors which can contribute to a suboptimal assay are nonspecific antibody binding or insufficiently cleaned flow cytometers. There is a lack of standardization of the denominator used for MRD enumeration. COG ALL-MRD assays maintain as denominator nucleated mononuclear cells [297], while others either relate MRD cells to total nucleated cells or total white cells, based on CD45 expression [396]. Dworzak et al. [347, 397] introduced the use of a live-cell-permeant nucleic acid-binding dye, such as Syto 16, a green fluorescent dye, to correct for nonnucleated cells and debris.

There are several other aspects of MRD determination, such as the choice of anticoagulant (EDTA or heparin is preferred) for the aspirate, use of cocktailed antibody combinations, and optimal ammonium chloride red cell lysis for white cell enrichment in the case of hypocellular marrow aspirates, a crucial step which is usually not paid attention to sufficiently. There are commercially available ACK (ammonium-chloride-potassium) lysing buffers and there are several home-brew recipes, which vary by temperature used during lysis (room temperature versus ice), speed during cell centrifugation after lysis, concentration of EDTA (ethylenediaminetetraacetic acid), and type of protein-containing buffer used to wash the white blood cells after lysis. I can only recommend that laboratories find the ACK buffer and lysing conditions which work best for their MRD assay, especially which avoid cell clumping.

The Two Philosophies of MFC-MRD Assays

MRD assays are widely applicable but require interpretive expertise. This notion is true whether MRD is based on leukemia-associated immunophenotypes (LAIP) [294, 303, 346, 382, 390, 396, 398, 399] established in the diagnostic specimen or the different-from-normal (DFN) approach [297, 301, 310, 343–345], which does not require knowledge of the presenting immunophenotype and relies on the recognition of cellular features that differ from those seen on normal cells of similar lineage and maturational stage. Cells that cluster in sites where normal cells are absent (the so-called empty spaces) are consequently defined as residual leukemic cells irrespective of the diagnostic phenotype of leukemia. Independent of the baseline phenotype, fixed antibody panels for leukemia population identification are used. Details regarding the methodology and antibody panels for all lineages have been published by B. Wood [399]. Which strategy to use is personal preference and a combination of the two might be the way to go. In fact, the contrast between these two philosophies appears to reflect more an individual conviction than actual scientific evidence. As Brent Wood [297] recently formulated, the use of LAIP is a simplified version of the DFN approach.

Preferentially, LAIPs consist of cell surface markers and combine antibodies which distinguish leukemic blasts from normal precursors; detect expression of lineage-foreign markers, e.g., lymphoid antigens on myeloblasts; detect altered density or lack of antigen expression; detect asynchronous expression of antigens (co-expression of antigens that are not concomitantly present during normal differentiation); or recognize antigens associated with particular genotypes (e.g., CD25 in BCR/ABL^{POS} ALL). Because of the dependence of LAIPs on the diagnosis immunophenotype, baseline and follow-up samples are ideally processed the same way and antibody combinations as well as fluorochrome choices for essential antibodies must be those used at diagnosis. LAIP-based MFC-MRD detection in the UK National Cancer Research Institute AML16 trial (NCT00454480) was performed in four reference laboratories with identical standard operating procedures, antibody panels, instrument type, and controls [400]. Still, these laboratories produced MRD values which were not identical. Especially in AML, multiple leukemic clones are frequently found requiring that equally multiple LAIPs be used in the MRD assay. However, it is recommended that only LAIPs expressed by a minimum of 10% of the leukemic blast population be monitored [401]. Though explained by the limits of sensitivity of MRD detection, this rule ignores the possibility that minor clones at presentation may dominate the remaining leukemia population at the time of MRD assessment or at relapse [282, 402]. Instability of even one of the LAIPs after treatment poses the risk of a false-negative MRD result.

The DFN-based analysis approach circumvents the problems of false negatives from phenotypic shifts and emerging subclones after treatment by using a fixed antibody panel. Most importantly, this approach avoids rigid gating predefined by the baseline specimen. Its independence from the diagnostic immunophenotype is very conveniently used in referral centers or commercial enterprises, when they receive samples for MRD determination without accompanying information on the baseline antigen profile. Both strategies are used in ALL and AML.

To reach satisfactory sensitivity against the background noise of antigen expression by normal bone marrow cells, leukemic immunophenotypes must be sufficiently aberrant to be present on less than 0.1 to 0.01% of normal lymphoid or myeloid marrow cells, so that in an adequate sample an MRD detection sensitivity threshold of 10^{-3} to 10^{-4} (1 in 1000 to 1 in 10,000 cells) can be achieved. However, for both LAIP and DFN methods, this signal-to-noise ratio is not a constant and varies with both treatment stage and leukemia subtype. The degree of abnormality in the population of interest is patient dependent. For leukemic phenotypes with a low degree of abnormality, the sensitivity of the MRD assay can be increased by acquiring more events (1,000,000 or even more) and the number of fluorochromes used per cell [303]. Furthermore, interference with the detection of MRD by normal cells will change with treatment stage. A simplified MRD assay in B-lineage ALL was based on the hypothesis that CD19^{POS}CD10^{POS}CD34$^{POS/NEG}$ cells detected early after initiation of treatment should be leukemic rather than normal, since normal bone marrow B-cell precursors (hematogones) with this phenotype are exquisitely sensitive to corticosteroids and thus eradicated during remission induction [403].

Hematogones and MFC-MRD Detection in B-lineage ALL

Hematogones are present in increased numbers during periods of hematopoietic regeneration, potentially causing interference with the detection of MRD [35, 36, 322]. In normal bone marrow, all differentiation stages of hematogones are present, whereby they exhibit a typical continuous differentiation spectrum that defines the normal evolution of B-cell precursors [35, 297, 322, 404]. This contrasts with discrete clusters of leukemic cells arrested at a certain stage of differentiation. In most studies, hematogones are differentiated from leukemic blasts by their expression pattern of CD45/CD19/CD10/CD20/CD38/CD22/CD34/CD58. As expected from normal B-lymphopoiesis, CD34 (and TdT) are present in the most immature stage of hematogones when CD10 is the highest and CD45 the lowest. With increasing maturation, hematogones lose CD10, and gain CD45, CD22, and some CD20. Zeidan et al. [405] demonstrated that the less mature hematogones express CD34 and lack CD123, whereas the more mature hematogones, with higher CD45 expression, lack CD34 but always express CD123. Given that the majority of B-ALL cases express both CD34 and CD123, this differential expression pattern was suggested to be helpful in distinguishing hematogones from leukemic blasts. As a side note, CD123 has been found to be overexpressed in B-lineage ALL patients with hyperdiploidy [351, 406]. It is also important to remember that leukemic lymphoblasts frequently express myeloid antigens, especially CD13 and/or CD33, which clearly allows the distinction from hematogones [407]. Based on differential gene expression between B-ALL and hematogones, Coustan-Smith et al. [351] found 22 markers which by flow cytometry were differentially expressed in the large majority of ALL cases tested, among them CD24, CD123, CD200, CD44, CD73, CD86, CD99, and BCL2. CD73 and CD86 have recently been confirmed as useful in differentiating B-lymphoblasts from hematogones [407]. While hematogones have been extensively studied in bone marrow, they are occasionally found in the blood (0.01–1.3%) according to Kroft et al. [408]. Chen et al. [409] identified a handful of proteins that were expressed in leukemic B-lymphoblasts at higher densities than in normal CD19^{POS}CD10^{POS}CD34^{POS} progenitors, including CD58 (LFA3), a ligand for CD2. Veltroni et al. [410] confirmed CD58 to be overexpressed by leukemic B-lymphoblasts when compared to regenerating and mature B-lymphocytes and no significant modulation of CD58 expression with treatment was noted.

While MRD detection in CD10^{POS} B-lineage ALL is plagued by the presence of hematogones, that in CD10^{NEG} B-ALL deals with the discrimination between leukemic cells and CD19^{POS}CD10^{NEG} plasma cells and plasmablasts. If one is lucky enough that the CD10^{NEG} leukemic blasts were CD34^{POS} at diagnosis, then CD34 negativity by suspicious cells together with high CD38 expression will definitely characterize those cells as plasma cells. CD38 expression by plasma cells exceeds by far that of leukemic B-lymphoblasts (or hematogones). CD138 (syndecan-1), a very useful marker of plasma cells, is usually not part of any MRD assay antibody panel. Plasmablasts, which may be found in marrow aspirates due to hemodilution, express CD38 at lower intensity than mature plasma cells, comparable to cases of CD10^{NEG} leukemic B-lymphoblasts, thus adding to a potential confusion. CD10^{NEG} pro-B or pre-pre-B-ALL blasts [44] vary in their expression of other B-cell differentiation antigens, such as CD22 and CD24, and CD20. Plasmablasts are CD20^{POS}, while plasma cells are CD20^{NEG}, CD24^{NEG}, but variably positive for CD22. Adding to a potential misinterpretation of CD10^{NEG} MRD is the infrequent finding of CD19 expression by natural killer cells [411]. Because MFC-based MRD identification is more difficult in CD10^{NEG} than CD10^{POS} ALL, standardized multicenter MRD testing demonstrated the highest discordant results between MFC- and PCR-MRD in CD10^{NEG} disease [348].

MFC-MRD Detection in T-lineage ALL

As mentioned before, the kinetic pattern of MRD response in T-lineage ALL is different from that in B-ALL with a slower rate of blast cell clearance in T-ALL, making end-of-induction MRD data less useful [379, 380]. Roshal et al. [412] reported that the main T-cell-associated antigens, surface CD3, CD2, CD4, CD5, CD7, and CD8, as well as CD45 remain relatively stable with treatment and thus can be reliably used in MRD detection. It is important to discriminate T-lymphoblasts from natural killer cells, which have variable expression of some T-cell antigens (especially CD7). Furthermore, the natural killer cell markers, CD16 and/or CD56, are occasionally expressed by T-ALL cells [413]. CD99 has been shown to be overexpressed in T-ALL when compared to hematopoietic stem cell and normal T-lymphocytes [412, 414, 415]. During T-lymphopoiesis, CD99 is downregulated with increasing expression of surface CD3 [416]. As a result, CD99 has been suggested as a very useful tool in MFC-MRD for T-ALL [414]. However, CD99 can be lost during therapy [412]. In fact, other markers of immaturity, such as TdT and CD34, dramatically decline during therapy of T-ALL as well [412].

MFC-MRD Detection in Myeloid Leukemias

Normal immunophenotypic patterns of maturation of hematopoietic lineages have been well described and they form the basis of MRD detection by the DFN approach [343]. While in lymphoid acute leukemias, the neoplastic population can be identified in most instances accurately even when present at very low levels, using either of the analysis approaches discussed, the situation is much more complicated in AML due to the heterogeneity of nonlymphoid acute leukemias and the multitude of differentiation stages to be encountered in follow-up samples [343]. A multitude of LAIPs have been proposed and utilized [346, 382, 417] and the DFN panel for MRD in AML has been published [399]. In APL, MFC-MRD determination is not very useful given the rather mature phenotype of leukemic promyelocytes and the ready availability of PCR-MRD monitoring for *PML/RAR*α transcripts. Theoretically, one could utilize the expression (albeit often weak) of CD117 and the differential expression of CD15s and CD15 by leukemic and normal promyelocytes, respectively [29]. Monocytic leukemias present the biggest challenge because leukemic monocytes show considerable overlap in antigen expression with normal monocytes. Van Lochem et al. [418] have suggested various immunostainings for studying aberrant monocytic differentiation, which, however, also delineate their potential pitfalls. The European LeukemiaNet reported that the most common aberrations observed in AML are $CD33^{POS}CD7^{POS}$ ($\pm CD34^{POS}$) and $CD34^{POS}/CD11b^{POS}$ ($\pm CD117^{POS}$) [395]. Together with low CD45 expression and side scatter characteristics, it was suggested that a combination of four or five surface markers could reliably detect MRD in AML. The biggest handicap to this LAIP-based MRD approach is the multitude of LAIPs often present at diagnosis and the challenge to monitor all of them. While the DFN-based MRD approach has the definite advantage of not worrying about presenting LAIPs and being flexible enough to detect phenotypic changes, there is still a substantial incidence (about 25%) of false-negative MRD reports even with this methodology [310].

That tumor heterogeneity in AML makes this disease a moving target for the detection of MRD has been well described [402]. Despite its genetic and biologic heterogeneity, a common most primitive cell population with the $Lin^{NEG}CD34^{POS}CD38^{NEG}$ phenotype was shown to be able to transfer AML to NOD-SCID mice [419]. Since these early studies, other markers have been detected on these LICs, including CD123, which is expressed by LICs at much higher levels than by normal hematopoietic stem cells [420]. Other differentiating markers of LICs are C-type lectin-like molecule-1 (CLL-1), CD96 (tactile), a member of the immunoglobulin gene family, CD47, the ligand for signal regulatory protein α, whose expression by LICs protects them from phagocytosis by macrophages and dendritic cells, and others [421]. However, there is marked variability in LIC antigen expression profiles among AML patients. LICs in *KMT2A(MLL)*-rearranged leukemias have variable surface antigen expression patterns dependent on gene fusion partners [8], also suggesting that the $CD34^{POS}CD38^{NEG}$ cell pool may not uniquely contains LICs. Most importantly for MRD detection, the LIC compartment in AML is more heterogeneous than previously anticipated and includes cells with the surface phenotype of committed progenitors [421, 422]. This suggests that measuring $CD34^{POS}CD38^{NEG}$ stem cells, as putative culprits of relapse, may not be entirely sufficient, although the frequency of these cells at diagnosis predicts for high MRD levels [423–426]. It is indeed surprising, though, that commonly used MFC-MRD assays, which solely measure residual cells from the original leukemic bulk, provide any clinically relevant information. The pre-leukemic stem cell clone, which has lost the major phenotype of bulk AML but contains the driver or founder mutations which led to the disease in the first place, survived standard treatment, thus persisted during remission, and eventually will lead to relapse (rev in [427]); this clone cannot (yet) be identified based on antigen expression. The mere evidence, however, that apparently mature myeloid cells in remission may be derived from leukemic progenitor cells [428, 429] makes MFC-based MRD measurements extremely difficult in AML.

Changes in Blast Cell Immunophenotypes with Treatment

Stability of LAIPs is a prerequisite for reliable LAIP-based MRD tracking. Despite the fact that differences in antigen expression levels between longitudinal studies during the treatment course of a patient may be due to technical aspects, like differences in analysis strategies or sample preparation, there is convincing evidence for true phenotypic changes. There is the reduction or disappearance of individual antigen expression and increments or gains in antigen expression; some phenotypes completely disappear while new abnormalities appear. The potential consequences of this phenomenon for MRD detection both in AML and ALL have been reviewed [402, 430]. Without question, changes in the immunophenotype of leukemic blasts with treatment and/or disease progression contribute to the approximately 25% of patients who relapse despite having been MRD$^{NEG/LOW}$ after induction or consolidation therapy.

Modern sequencing techniques have allowed us to prove beyond any doubt that the genetic landscape of leukemic subpopulations in a patient continues to evolve, either due to natural progression of the disease or in response to selective pressure from treatment [431, 432]. It is therefore not surprising if changes in surface phenotypes are observed between presentation and relapse but also early on during induction chemotherapy. Gaipa et al. [433] found the downregulation of CD10 and CD34, while CD19, CD11a, and CD20 were upregulated during the initial phase of induction treatment for pediatric B-lineage ALL, indicative of progressive maturation. These changes, which were found to be caused by glucocorticoid administration, were transitory. Remarkably, normal B-lymphocytes present in the specimens were equally affected. Rather than due to subclone selection or apoptosis-related artifacts, these changes (except for CD11a) were a direct result of drug-induced modulation [434]. Interestingly, the extent of phenotypic shift correlated positively with sensitivity to drug treatment [433]. Nonetheless, phenotypic shifts of highly relevant MRD antigens, like CD10 and CD34, returned to their initial aberrant expression levels after glucocorticoid-containing therapy was discontinued [434]. In addition, phenotypic modulation creates new combinations of aberrant expressions during follow-up, in particular based on upregulation of CD20. Genome-wide gene expression analysis revealed that glucocorticoid administration for 8 days resulted in decreased proliferative activity of the blast cells and a differentiation shift towards normal B-cells, both developments which may be the cause for the persistence of these blasts at day 8 of therapy as a result of drug resistance [435]. Rhein et al. [435] also reported increased expression of CD11b (MAC-1) integrin and CD119 (interferon gamma receptor-1) on day 8 B-lymphoblasts. The expression of CD11b in B-lineage ALL was found to confer a high risk of MRD when present on B-lymphoblasts at diagnosis [133]. Similar to B-lineage ALL, also leukemic T-lymphoblasts lose markers of immaturity during therapy, such as CD34, TdT, and CD99 [412].

Borowitz et al. [430] reviewed diagnostic and relapse immunophenotypes in children with ALL and found that although phenotypic shifts are common, they do not interfere with MRD detection, especially when the DFN approach is used. Of interest, these authors reported that the MRD-positive population at day 29 resembled the diagnostic specimen in 63% of cases, whereas in one-third of patients the day 29 MRD resembled the relapse phenotype, and in 2 out of 29 cases, day 29 MRD was unrelated to either phenotype. Surprisingly, time to relapse was identical in these groups. Contrary to what has been observed in ALL, phenotypic changes from diagnosis in AML point to a more immature state, through loss of CD11b and CD15 and frequent gain of CD34 and CD117 [298, 402]. A unique occurrence has been reported by Slamova et al. [436], a switch from CD2^{POS} B-ALL to acute leukemia of the monocytic lineage, associated with demethylation of the CEBPα gene and upregulation of this gene.

Epigenetic diversity within the LIC population and its progeny is an interesting alternative to permanent genetic modification as a cause of phenotypic changes, given that epigenetic modifications are dynamic and transient and allow the epigenetic status to return to its original status after selective pressure has been overcome. Although the actual mechanisms by which phenotype changes occur are unclear, the two most favored options are (1) spontaneous gains or loss of mutations in the primary tumor clone(s) present at the time of diagnosis and occurring during or after therapy and (2) selection of minor therapy-resistant subclones, already present but not routinely detected at diagnosis, which survived therapy and grew out to cause relapse [431].

With the increase in monoclonal antibody therapy, flow cytometrists face a novel situation, the potential of antigen loss or downregulation due to therapeutic antibody exposure. In patients treated with gemtuzumab ozogamicin, CD33 expression persisted at relapse in most cases [437]. In contrast, after rituximab therapy, more than one-third of cases lost CD20 expression [438]. With the bi-specific CD3/CD19-targeting antibody, blinatumomab, loss of CD19 expression occurred with treatment and at the time of relapse [439]. The mechanisms of CD19 loss after CD19-directed therapy have been discussed before.

As true for flow cytometry in general, MFC-based MRD determination cannot function in a vacuum, isolated from all other aspects of leukemia biology. Mooreman et al. [440] demonstrated that a subgroup of genetically good-risk ALL patients remained MRD^{POS} after induction, which was associated with more incidences of relapse but an overall survival of >90%. Rather than superseding other prognostic factors [441], MRD complements the power of genetic factors for risk stratification of patients with acute leukemia.

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Cytogenetics of Acute Leukemia

16

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Introduction

Cytogenetic abnormalities are an important factor in patient diagnosis, risk assessment, and treatment in acute leukemia. Characteristic genetic abnormalities are recognized as essential for disease classification and are a significant element of the 2008 World Health Organization (WHO) classification [1]. Some aberrations are involved in the initiation whereas others participate in the progression of different acute leukemias. Cytogenetic aberrations occur in most malignant cells, revealing acquired genomic changes that may have diagnostic and prognostic significance. This chapter reviews the most important cytogenetic and molecular genetic lesions in acute myeloid leukemia (AML) and acute lymphoblastic leukemia (ALL).

In acute leukemias, the bone marrow is the sample of choice to detect cytogenetic aberrations in malignant cells because cells of the bone marrow divide spontaneously; however, peripheral blood may also be studied if there is a significant number of circulating blasts. It should be noted that normal chromosomes in the periphery do not necessarily reflect a cytogenetically normal marrow because reactive rather than malignant cells may be dividing. Cytogenetic analysis involves either a direct procedure or culture of cells overnight to obtain dividing cells, which are necessary to observe chromosomes. Cells are exposed to colchicine to arrest the cells in division, when chromosomes are sufficiently contracted for visualization. The cells are fixed, banded, and analyzed. As only 20 completely analyzed metaphases are required for a cytogenetics evaluation, metaphase cytogenetics is limited in its sensitivity to detect abnormalities.

The sensitivity of cytogenetics has been greatly enhanced by the application of fluorescence in situ hybridization (FISH). FISH has notably advanced the field of cytogenetics by enabling the detection of numerical and structural aberrations in both hematologic malignancies and solid tumors. FISH is a molecular cytogenetic technique which uses a DNA probe to evaluate cells in either metaphase or interphase. Various types of probes are commercially available and are applied to detect gains, losses, and different types of cytogenetic rearrangements. The probes vary in size and can detect aberrations as small as 200 kb, although most clinically applied probes are much larger. This is in contrast to metaphase cytogenetics, in which most aberrations must be at least 2 Mb for detection. Technically, both the probe and the chromosomes are "denatured" (made single stranded), hybridization of the fluorescently labeled probe to its matching DNA on the chromosomes is allowed to occur, the excess probe is removed with several washings, and the preparations are analyzed using a fluorescence microscope. In addition to detecting much smaller aberrations, FISH is more sensitive than metaphase analysis because 200 interphase cells are generally analyzed. Furthermore, dividing cells are not required, so neither culturing nor live cells are necessary as for metaphase cytogenetics, making application to a wider range of tissues possible.

Different FISH probe "strategies" are used to detect different types of abnormalities. The ones commonly used in analysis of acute leukemias include probes for detection of rearrangements, gains, and losses. These include probes designed to detect specific loci, such as *TP53*, to detect centromeres, and to detect whole chromosomes (chromosome paints—metaphase analyses). The use of dual-color dual-fusion probes is another strategy to detect translocations. The breakpoints of each of the translocation partners are labeled in different colors, generally a red and a green, such that both probes are split when there is a translocation. Fusion of the two probes indicates the rearrangement, and since most

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translocations are reciprocal, two fusions are typically present in addition to the two normal alleles. Thus, the pattern observed in abnormal cells with the translocation queried is two fusions, one red and one green signal. Break-apart probes have been designed to detect rearrangements of genes that frequently have different partners, such as *KMT2A* (formerly *MLL*). These probes are labeled with one color 5′ of the breakpoint, and a second color 3′ of the breakpoint, yielding two fusion signals in a normal cell. If the gene has been rearranged, one signal is split and one red, one green, and one fused signal will be detected in abnormal cells. In many cases, FISH should be performed on metaphase chromosome preparations, because there are many variations of each genetic abnormality at the DNA level and because analysis of only interphase nuclei can lead to misinterpretation of results. Furthermore, FISH analysis has shown that in several cases "balanced" translocations detected by conventional cytogenetics are not actually balanced, but rather associated with submicroscopic deletions. However, the clinical significance of these deletions remains unknown [2]. Use of FISH has become very common in the cytogenetic laboratory, as it is rapid and precise and gives a quick and accurate diagnosis. Because of its sensitivity, FISH is also useful to detect cytogenetically cryptic aberrations, such as the t(12;21)(p13;q22) found in ~25% of pediatric ALL. It also is frequently utilized to help define a complex karyotype or to confirm an abnormality suspected in a karyotype. However, FISH can only give results concerning the specific probes applied; the presence or absence of other aberrations is not detected.

Conventional cytogenetic methods, FISH and reverse transcriptase-polymerase chain reaction (RT-PCR), are complementary and can reliably identify clonal rearrangements of genes and aid in subclassification of disease subtypes in acute leukemias [3]. RT-PCR may be a useful adjunct to FISH when cytogenetic analysis is not possible because of a lack of dividing cells. For example, the cryptic t(12;21) generates an *ETV6–RUNX1* chimeric fusion that is easily identified by FISH or RT-PCR. Cytogenetic analyses of t(8;21), inv(16), or t(15;17) in patients with AML yield a low rate of false positives, but false negatives occur in rare cases when cryptic, complex rearrangements are not detected [4, 5]. Thus, it is clinically important to use FISH and RT-PCR separately or in combination to detect cryptic abnormalities, especially for disease subtypes such as acute promyelocytic leukemia (APL) wherein optimal therapy differs.

In some subgroups it may be difficult or not feasible to detect all translocations because of the diversity of partners (e.g., *KMT2A* with multiple partners) or because the genes involved are located near the telomeric regions of the chromosomes [e.g., *NUP98*(11p15.5), *RUNX1*(21q22), *ABL1*(9q34.1), *JAK2*(9p24), *ETV6*(12p13.2)]. For *KMT2A* cases, a long-distance inverse PCR method has been successfully used to identify any type of *KMT2A* rearrangement at the molecular level [6]. The rapid amplification of cDNA end PCR method can also be used to clone breakpoints of partner genes such as of *KMT2A*, or of *PDGFRB* in chronic myeloproliferative disorders or of *NUP98* in myeloid leukemias.

At present, newly developed methods such as array-comparative genomic hybridization (aCGH) and single-nucleotide polymorphism (SNP) arrays quantitatively analyze DNA copy number at high resolution and systematically detect changes on a genomic scale. The implementation of these technologies has improved the resolution with which genetic alterations, especially changes in copy number, can be localized to the human genome [7]. With novel emerging technologies aimed to genetic, immunophenotypic, epigenetic, and proteomic classification, as well as next-generation sequencing (NGS) approaches to aid the identification of new molecular subsets, the classification of acute leukemias will likely evolve to provide informative prognostic and biologic guidelines to clinicians and researchers.

Acute Myeloid Leukemia

AML, a malignant disorder of the bone marrow, develops because of the clonal transformation of a multipotent stem cell through the acquisition of chromosomal rearrangements and multiple mutations. Cure rates for AML have improved only moderately over the past few decades compared with those for ALL. Clonal, nonrandom chromosomal abnormalities have been observed by conventional cytogenetics in blast cells of 80% of children and adolescents and 60% of adults with AML.

Cytogenetic analysis is a primary component in the diagnosis and treatment of AML. According to the World Health Organization classification (WHO-2008) [1], results of diagnostic studies should correlate with clinical findings and be reported in a single, integrated report that includes the cytogenetic profile. In addition, a minimum of 20% blasts is typically required for the disease to be classified as AML. However, with t(8;21), inv(16)/t(16;16), and t(15;17), the disease is considered AML even if less than 20% blasts are present. WHO-2008 expanded the number of recognized chromosomal abnormalities associated with AML and for the first time included specific gene mutations (*CEBPA* and *NPM1)* as provisional categories [1] (Table 16.1). *FLT3* internal tandem duplication (*FLT3*-ITD) is included because of its recognized prognostic significance. In addition to cytogenetic abnormalities, with the advent of powerful high-throughput tools, these and other recurrent genetic mutations have been incorporated into clinical practice. A revision of the WHO classification

Table 16.1 World Health Organization 2008 classification of acute myeloid leukemias with recurrent genetic abnormalities

AML with t(8;21)(q22;q22)/*RUNX1-RUNX1T1* (*AML1-ETO*)
AML with inv(16)(p13.1q22) or t(16;16)(p13.1q22)/*CBFB-MYH11*
APL with t(15;17)(q22;q21)/*PML-RARA*
AML with t(9;11)(p22;q23)/*MLLT3 -MLL* (specify other 11q23/*MLL* abnormality)
AML with t(6;9)(p23;q34)/*DEK-NUP214*(*CAN*)[a]
AML with inv(3)(q21q26.2) or t(3;3)(q21;q26.2)/*RPN1-MECOM*[a]
AML with t(1;22)(p13;q13)/*RBM15-MKL1*(*OTT-MAL*)[a]
Provisional entity: AML with mutated *NPM1*[a]
Provisional entity: AML with mutated *CEBPA*[a]

AML acute myeloid leukemia; *APL* acute promyelocytic leukemia
[a]Newly defined entities by WHO-2008 [1]

is under way to include the many lesions identified by genomic methods, which are either targetable for therapy or useful for prognostication. This update is expected to improve the risk classification for AML [8].

Favorable prognostic factors for patients with AML include low white blood cell (WBC) count; inv(16)/t(16;16); t(8;21); t(15;17); *NPM1* mutation (in the absence of *FLT3*-ITD or *DNMT3A* mutation); biallelic *CEBPA* mutation; Down syndrome (DS) in children up to 4 years old, mostly with acute megakaryoblastic leukemia (AMKL, AML-M7); and early response to treatment. Unfavorable prognostic factors include –7, –5/5q, t(6;9)(p23;q34), t(6;11)(q27;q23), and 3q abnormalities; complex karyotypes (≥3 unrelated cytogenetic abnormalities, excluding the categories described by WHO-2008); cryptic lesions mostly observed in children such as t(5;11)(q35;p15.5)/*NUP98–NSD1*, t(6;11)(q27;q23)/*KMT2A–MLLT4(MLL–AF6)*, inv(16)(p13.3q24.3)/*CBFA2T3–GLIS2*, t(11;12)(p15.5;p13.3)/*NUP98–KDM5A*, and t(7;12)(q36.3;p13.2)/*MNX1–ETV6*; as well as lesions found in all age groups, 17p/*TP53* deletion or mutation; *KMT2A*(*MLL*)-PTD and *FLT3*-ITD, particularly with a high allelic ratio; and *DNMT3A, ASXL1,* and *RUNX1* mutations. Intermediate-risk factors include normal and other karyotypes and genetic lesions excluding favorable and unfavorable abnormalities [1, 9–12].

AML represents 15–20% of all childhood leukemias and approximately 33% of adolescent and 50% of adult leukemias. The prognostic value of cytogenetics has been well established for all age groups [9, 10, 12, 13]. However, the distribution of recurrent chromosomal abnormalities observed in AML differs by age groups. In infants (<2 years old), chromosomal abnormalities most frequently include a breakpoint in 11q23 and involve *KMT2A*(*MLL*) in 60% of cases. The incidence decreases to 30% in children (2 to <13 years) and to 13% in adolescents (13 to <21 years), and is even lower in young adults (8%; 21 to <30 years) and adults (3%; ≥30 years) [13]. The t(1;22)(p13;q13) is specific for AMKL and is mostly seen in children younger than 2 years and rarely in adults. In older children (>10 years) and young adults (<60 years), the most frequent abnormalities are t(15;17), t(8;21), and inv(16). In older patients (>60 years) with AML, there is a low frequency of favorable core-binding factor chromosomal abnormalities but a higher incidence of complex aberrant karyotypes, which is attributed to both disease biology and host factors [14]. A previous study evaluating the effect of age on outcome of AML in children 21 years or younger suggested that age is an independent prognostic factor in childhood AML and that children younger than 10 years benefit more than older children from newer intensive therapies [15].

The following section describes the associations among specific recurring cytogenetic and molecular abnormalities in AML, distinct clinical subtypes of diseases, and treatment outcomes.

Specific Recurring Cytogenetic and Molecular Abnormalities

t(15;17)(q24;q21)/*PML–RARA*

The t(15;17)(q24;q21) is associated with APL, a distinct subtype of AML that is treated differently from other types of AML because of its marked sensitivity to the differentiating effects of all-*trans* retinoic acid (ATRA). The t(15;17) is found in 9% of children with AML; it is rarely observed in infants, and the incidence decreases with age in adults (rarely seen in adults 45 years or older) (Fig. 16.1). The t(15;17) generates the fusion gene *PML–RARA* to produce a chimeric protein. Patients with the t(15;17) and APL respond well to the combination therapy of ATRA and the chemotherapy agent arsenic trioxide (ATO) and have a favorable prognosis (cure rate 80%) [16–19].

A suspected diagnosis of APL should be treated as a medical emergency because immediate measures are needed to counteract coagulopathy and initiate ATRA therapy. Thus, a prompt genetic diagnosis by conventional cytogenetics, FISH with *PML–RARA* probes, RT-PCR, or anti-PML monoclonal antibodies, is essential for patients with suspected APL [18]. *Conventional G-banding* has helped to identify the t(15;17) in more than 90% of patients with APL, but it cannot detect the *PML–RARA* fusion resulting from cryptic rearrangements. However, cytogenetics facilitates the identification of rare variant translocations, including three-way translocations affecting *PML* and *RARA* or other aberrations affecting only *RARA*. FISH with commercially available probes is a rapid, highly sensitive, and specific method to confirm the presence of the *PML–RARA* fusion. However, in rare instances, fusion

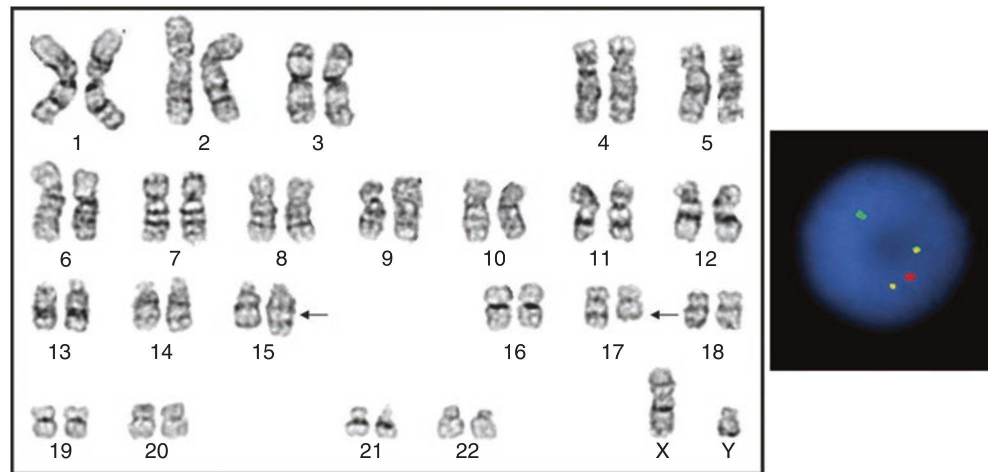

Fig. 16.1 *Left*: Karyogram of a cell from a patient with APL and the typical translocation between chromosomes 15 and 17 (*arrows*). The karyotype is 46,XY,t(15;17)(q24;q21). *Arrows* indicate abnormal chromosomes. *Right*: FISH with the dual-color dual-fusion *PML–RARA* probes (Abbott Molecular) showing 2 fusion (*yellow*), 1 *red*, and 1 *green* signals

signals might be different from those expected, because of deletion of sequences at translocation junctions or where *PML–RARA* is formed as a result of an insertion or a complex translocation [5]. FISH using *RARA* break-apart probes is useful to evaluate rearrangements in suspected cases that lack the *PML–RARA* fusion. *RT-PCR* is important to establish the type of *PML–RARA* isoform fusion transcript that will be the target to reliably monitor minimal residual disease. *Immunostaining* with anti-PML monoclonal antibodies on dry smears is also useful to establish a rapid diagnosis.

Small *PML–RARA* insertions can be missed by FISH when using very large probes; in such cases, it is more appropriate to use relatively small cosmid probes. Patients with APL in whom the *PML–RARA* fusion is generated due to variant three-way translocations or cryptic insertions respond to ATRA combination regimens in a similar manner as those with APL with the typical t(15;17) [5].

Other rare translocations involving *RARA* can also result in APL. Approximately 1% of patients with APL have variant translocations involving only *RARA*. The most common is t(11;17)(q23;q21)/*PLZF*(*ZBTB16*)–*RARA* (~20 patients) [20]. These patients and another patient with a rare inv(17)(q11.2q21)/*STAT5B-RARA* were partially resistant to ATRA [21]. Other variant translocations with *RARA* include *OBFC2A (NABP1)* (2q32), *TBL1XR1* (3q26), *FIPIL1* (4q12), *NPM1* (5q35), *NUMA1* (11q13), *ZBTB16* (11q23), *PRKAR1A* (17q24.2), and *BCOR* (Xp11.4) [20, 22, 23]. It is important to identify patients with such translocations, as the sensitivity for ATRA or ATO may vary.

Children with APL have a higher incidence of hyperleukocytosis (WBC count $\geq 10 \times 10^9$/L) than adults with APL, but the high WBC count is associated with poor outcome in both groups [18, 19]. *FLT3* mutations [either internal tandem duplications (ITDs) or kinase domain mutations] occur in up to 40% of patients with APL and are associated with high WBC counts; however, there is no clear correlation between the presence of *FLT3* mutations and outcome [19, 24, 25].

Core-Binding Factor Leukemias

In children and adults with AML, the highest complete response rates and longest survival times are associated with the two common aberrations, t(8;21) and either inv(16) or t(16;16). Both aberrations disrupt the core-binding protein complex, which plays a vital role in hematopoiesis, and are designated core-binding factor (CBF) leukemias. The t(8;21) targets *RUNX1*, and the inv(16)/t(16;16) targets *CBFB*. Several studies have shown an association between cooperating mutations and CBF leukemia; the most prevalent are *KIT* and *NRAS* mutations, but their prognostic significance remains debated [26].

t(8;21)(q22;q22)/*RUNX1*(*AML1*, *CBFA2*)-*RUNX1T1*(*ETO,MTG8*)

The t(8;21)(q22;q22) is among the most frequent recurrent abnormalities in AML (12% of children and 6% of adults). The t(8;21) juxtaposes *RUNX1* to *RUNX1T1* on the derivative chromosome 8, generating a chimeric gene *RUNX1–RUNX1T1* (*AML1–ETO*), which inhibits transcriptional activation by wild-type *RUNX1*. RT-PCR and *RUNX1–RUNX1T1* FISH probes are available to detect the t(8;21) and are useful especially when submicroscopic rearrangements are suspected or the sample does not yield metaphases for analysis. Additional cytogenetic aberrations are present in 68% of patients with t(8;21). The most frequent additional change in cases with t(8;21) is loss of a sex chromosome in approximately 40% of patients; the Y chromosome is lost in males more frequently (55%) than the X chromosome (33%) in females [27, 28]. A del(9q), rearrangement of 7q, +4, or +8, can also occur, but does not significantly affect the overall survival. However, a del(9q) was associated with lower complete remission and +4 with inferior outcome in a recent study [27]. The t(8;21) is associated with a favorable

outcome, especially when the disease is treated with regimens containing high-dose cytarabine [27].

Presence of the *RUNX1–RUNX1T1* transcript in the bone marrow in adults and cord blood cells of children who do not have AML suggests that the t(8;21) is generated early in hematopoiesis. Some people may acquire secondary genetic alterations that lead to the development of AML [29], which is supported by the detection of *RUNX1–RUNX1T1* in neonatal blood spots of infants in whom AML subsequently developed (after >10 years) [30]. The most frequent genetic lesions identified are *KIT, ASXL2,* and Ras-pathway mutations [31].

inv(16)(p13.1q22) and t(16;16)(p13.1;q22)/*CBFB-MYH11*(*SMMHC*)

The inv(16) and t(16;16) result in fusion of *CBFB* at 16q22 to the smooth muscle myosin heavy-chain gene (*MYH11*) at 16p13.1. *CBFB* is the heterodimeric partner of *RUNX1*, which together form the transcriptional activating factor designated as CBF. The inv(16)/t(16;16) is strongly associated with the FAB M4Eo subtype and is observed in approximately 6% of children and adults with AML. FISH probes and RT-PCR are available to evaluate the status of fusion genes in patients with AML and can aid in the diagnosis of this subtle chromosomal abnormality. Additional abnormalities in leukemic cells with an inv(16) are found in up to 40% of patients, with +22, +8, and +21 being the most frequent. The inv(16)/t(16;16) confers a good prognosis in both children and adults with AML. The most frequent genetic lesions identified with inv(16)/t(16;16) are *KIT*, Ras-pathway mutations, and *FLT3*-ITD [32].

Other *RUNX1*(*AML1*,*CBFA2*) (21q22) Rearrangements

The *RUNX1* gene encodes a transcription factor that is important for hematopoiesis and is one of the most frequently mutated genes in acute leukemias. Chromosomal abnormalities include the t(12;21)(p13;q22)/*ETV6–RUNX1*(*TEL–AML1*) in ALL and many other *RUNX1* partner genes identified in AML, but several of them are reported for single cases (<1% of pediatric AML). One of the most frequently identified *RUNX1* partner genes is *MECOM(EVI1,MDS1)*, involved in the t(3;21)(q26.2;q22) associated with therapy-related MDS and CML in blast crisis [33]. The t(3;21) can generate fusion of *RUNX1* with several genes such as *EVI1, EAP*, and *MDS1*. In addition, other rearrangements of 3q26 (such as inv(3)(q21q26)) often lead to overexpression of *EVI1*, which is associated with poor prognosis, suggesting its role in the pathogenesis of AML [34]. Determination of *EVI1* expression is likely important, because it is overexpressed in cases with cryptic 3q26 rearrangements [35]. Another rare but recurrent translocation is the t(16;21)(q24;q22), mostly observed in patients with therapy-related acute leukemias and MDS but also in children and adults with de novo AML. The t(16;21) generates the fusion gene *RUNX1–CBFA2T*(*MTG16*), which is similar to the *RUNX1–RUNX1T1* generated by the t(8;21). Of interest, a t(16;21)(p11;q22)/*FUS–ERG* is a rare recurrent abnormality associated with poor prognosis in AML. *RUNX1* mutations have been identified in AML with noncomplex karyotype and confer an unfavorable prognosis [36].

Other 3q26.2/*MECOM*(*EVI1*, *MDS1*) and 3q21.3/*GATA2*/*RPN1* Rearrangements

These aberrations are more frequently observed in adults with AML than in children with AML and are adverse prognostic factors [37, 38]. *EVI1*-rearranged AML is characterized by distinct molecular alterations [38, 39]. The molecular basis of inv(3)(q21q26.2) and t(3;3)(q21;q26.2) (observed frequently with –7) has been revised with the rearrangement of a *GATA2*(3q21.3) oncogenic enhancer element rather than of the *RPN1* gene with *MECOM(EVI1)* [8, 40]. Of interest, a recent study identified *GATA2* mutation as the most common germline defect predisposing to pediatric MDS, with a very high prevalence in adolescents with –7, but did not confer poor prognosis [41].

11q23/*KMT2A* (*MLL*, *MLL1*, *ALL1*, *TRX*, *HTRX1*)

Most myeloid blasts with an 11q23 abnormality have a *KMT2A* rearrangement and are associated with monocytic differentiation. Every *KMT2A* translocation results in gain of function by generating novel chimeric proteins coded by the N-terminus of *KMT2A* fused in-frame with one of many partner genes coding for diverse functions, and each oncogene is associated with a distinct form of leukemia. More than 120 translocations target *KMT2A* in acute leukemias, and approximately 80 partner genes for *KMT2A* have been cloned [42, 43]. Despite the diversity of chimeric proteins created, *KMT2A* fusions generate a characteristic gene expression profile, including upregulation of developmental homeobox genes such as *HOXA9* and *MEIS1*, suggesting that partner genes for *KMT2A* might be involved in regulating transcription elongation and chromatin structure remodeling.

Chromosomal rearrangements involving *KMT2A* in AML and in ALL include balanced and unbalanced translocations, inversions, insertions, amplifications, and partial tandem duplications (*KMT2A*(*MLL*)-PTD). These genetic

mechanisms convert *KMT2A* into a chimeric transcription factor with leukemogenic properties. Some *KMT2A* gene rearrangements are not detected by conventional cytogenetic methods. In other *KMT2A* rearrangements, the precise breakpoint or gene partner is difficult to assign because different genes are mapped on chromosome regions with limited banding resolution, such as 10p and 19p [42]. FISH using commercially available dual-color *KMT2A* probes allows evaluation of derivatives of a translocation involving *KMT2A* in metaphase chromosomes and the splitting of signals in interphase nuclei (Fig. 16.2). In rare instances, FISH with this probe detects not only the reciprocal translocation but also a deletion of at least 190 kb from the 3′ region of *KMT2A*.

A *KMT2A* rearrangement is observed in up to 25% of children with AML, is prevalent in infants (~60%), and declines to less than 10% in older children with a median age of ~2 years. Pediatric patients with t(6;11)(q27;q23) and t(11;17)(q23;q21) are significantly older at presentation (12 years and 9 years, respectively). In contrast to the poor prognosis of infants with 11q23/*KMT2A*⁺ ALL, the outcome of infants with 11q23/*KMT2A*⁺ AML is not poorer than that of infants who do not have this rearrangement. The specific fusion partner of *KMT2A* may influence prognosis [44]. WHO-2008 has classified the t(9;11) as a separate category and recommends that partner genes be determined for patients with *KMT2A* rearrangements [1]. Several studies have reported a more favorable prognosis for children with t(9;11)(p22;q23)/*KMT2A-MLLT3* (50% of patients with *KMT2A* rearrangements) than children with other *KMT2A* rearrangements, but other studies have refuted these observations.

In a recent collaborative group study of 756 children with AML and an 11q23/*KMT2A* rearrangement, the overall 5-year event-free survival (EFS) was 44% [44]. The outcome of the subgroups varied greatly; for example, patients with a t(1;11)(q21;q23) (~3% of patients with *KMT2A* rearrangements) had an excellent outcome (5-year EFS, 92%), whereas those with a t(6;11)(q27;q23) (~9%) had the worst outcome (5-year EFS, 11%). Subgroups with a t(10;11)(p12;q23) (15%), t(4;11)(q21;q23) (<1%), and t(10;11)(p11.2;q23) (<1%) had 5-year EFS of 31%, 29%, and 17%, respectively [44]. A follow-up study by the international group reported that additional chromosomal abnormalities further influenced the outcome of children with *KMT2A* rearrangements, with complex karyotypes and trisomy 21 predicting poor outcome and trisomy 8 predicting a more favorable outcome [45].

A *KMT2A*-PTD is detected in approximately 10% of patients with AML, particularly those with trisomy 11 or a normal karyotype, and confers a poor prognosis [46]. In the 1990s, the use of epipodophyllotoxins was associated with the development of therapy-related AML (t-AML) with 11q23 abnormalities. The most frequently observed translocations were t(9;11)(p22;q23), t(11;19)(q23;p13.3), and t(11;16)(q23;p13.3). The t(11;16)(q23;p13.3), affecting *CREBBP*(*CBP*), was rarely seen in de novo AML. Subsequently, the dose and schedule of topoisomerase II inhibitors for treatment of acute leukemias were modified, which has reduced the occurrence of t-AML with a *KMT2A* rearrangement. Leukemias with *KMT2A* rearrangements are sensitive to BET, hDOTIL, and CDK6 inhibitors, which are currently being tested in an early clinical trial [12].

t(10;11)(p12;q14)/*PICALM*(*CALM*)–*MLLT10*(*AF10*)

The t(10;11)(p12;q14) is a rare recurrent abnormality seen in both adult and pediatric patients with AML or T-ALL and is often associated with a poor prognosis [47–49]. The t(10;11)(p12;q14) lacks a *KMT2A* rearrangement and can be difficult to differentiate microscopically from the t(10;11)(p12;q23)/*KMT2A–MLLT10*, and FISH or RT-PCR analysis is usually required to distinguish these two translocations with accuracy.

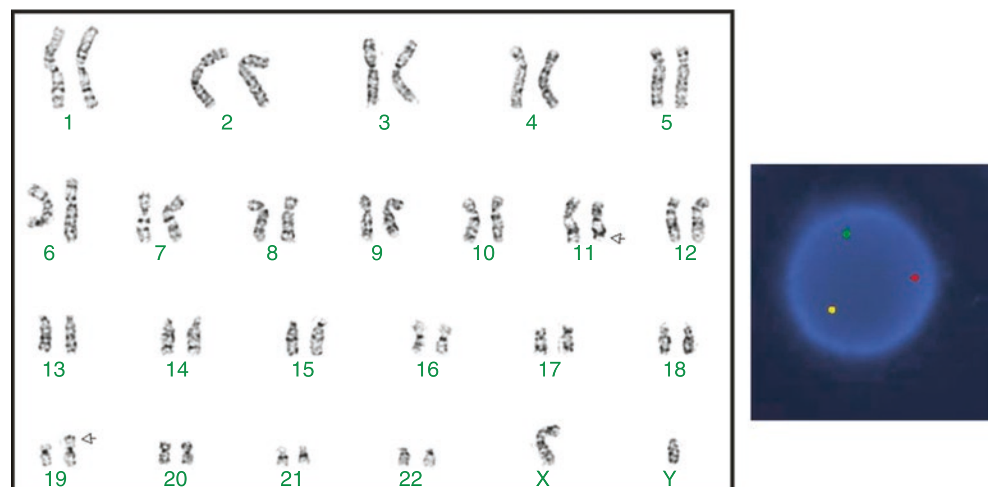

Fig. 16.2 *Left*: Karyogram of a cell with a t(11;19)(q23;p13.3), which occurs in both AML and ALL. *Arrows* indicate abnormal chromosomes. The karyotype is 46,XY,t(11;19)(q23;p13.3). *Right*: FISH with a *KMT2A* break-apart probe (Abbott Molecular) shows the separation of the 2 *KMT2A* signals (1 *red* and 1 *green*) and a normal fused (*yellow*) signal indicating a rearrangement of the *KMT2A* gene

t(1;22)(p13;q13)/*RBM15*(*OTT*)–*MKL1*(*MAL*)

The t(1;22)(p13;q13) is restricted to patients with AMKL and occurs in 33% of this subgroup, mostly in infants and young children. The t(1;22)(p13;q13) is rare in children with DS who develop AMKL. The t(1;22) juxtaposes the *RBM15(OTT)* on 1p13 to *MKL1(MAL)* on 22q13. Patients with this translocation present with hepatosplenomegaly and bone marrow fibrosis and can have high-hyperdiploid karyotypes. These patients were earlier classified as high risk, but the use of intensive therapy has significantly improved the prognosis [50–52].

t(3;5)(q25;q35)/*NPM1–MLF1*

The t(3;5)(q25;q35) is rare in young patients with AML and can present with trilineage dysplasia. However, patients with the t(3;5) have good outcomes [53, 54].

t(6;9)(p23;q34)/*DEK–NUP214*(*CAN*)

This translocation leads to formation of a leukemia-associated fusion protein DEK–NUP214(CAN), which occurs in up to 2% of children with AML. It is associated with relatively late onset (median age, 10.4 years) and male predominance. The t(6;9) presents with trilineage dysplasia and bone marrow basophilia and is the sole abnormality in 80% of the patients; the most common secondary changes are +8 or +13. The t(6;9) is strongly associated with *FLT3*-ITD mutations (up to 70% of patients), and has a poor prognosis [55]. Outcomes might improve with stem cell transplantation [56, 57].

t(X;6)(p11.2;q23.3)/*GATA1-MYB*

The t(X;6)(p11.2;q23.3) generates an *MYB–GATA1* fusion. The translocation is very rare, with few cases reported worldwide. It is predominantly seen in male infants presenting with basophilic leukemia, and most patients were in long-term complete remission at the time of reporting [58].

Recurrent Abnormalities of Chromosomes 5 and 7

Cytogenetic aberrations associated with unfavorable outcomes include monosomy 5 (−5) and del(5q)/5q–, monosomy 7 (−7), and complex abnormalities. These abnormalities occur more frequently in adults than in children. Patients with del(7q)/7q– are no longer considered to be at high risk of treatment failure [59]. Monosomy 7 is observed in approximately 2% of children with AML. The majority (72%) of adolescents with MDS and –7 carry an underlying *GATA2* deficiency, which does not confer poor prognosis [41]. In a large pediatric series, the prevalence of –5/del(5q) was 1.2%, the abnormality occurred in older children (median age, 12.5 years), it had a high incidence of undifferentiated blast morphology, and most patients had additional chromosomal abnormalities. The study confirmed the very poor prognosis of children with a –5/5q– [60].

t(8;16)(p11.2;p13.3)/*KAT6A*(*MOZ/MYST3*)–*CREBBP*(*CBP*)

Disruption of *KAT6A* at 8p11.2 is rare (~1%) and is seen in children (median age, 1.2 years), younger adults (median age, 45 years), and t-AML. It is associated with a poor prognosis [61]. The most frequent translocation is t(8;16)(p11.2;p13.3), which fuses *KAT6A* to *CREBBP*. Blast cell morphology is similar to that of acute myelomonocytic or monocytic leukemia, with the blast cells frequently (70%) displaying erythrophagocytosis. Patients with t(8;16)(p11.2;p13.3) can present with leukemia cutis (58%) and disseminated intravascular coagulation (39%); the coagulopathy may mimic that seen in APL [62, 63]. Importantly, spontaneous remissions occur in a subset of neonatal patients [28% of patients with a t(8;16) are diagnosed in the first month of life] and warrant a watch-and-wait strategy before initiating therapy [62]. The t(8;16) has a unique gene expression profile that clusters close to 11q23/*KMT2A* [62, 63]. Other *KAT6A* fusion partners are *NCOA2*(*TIF2*) in inv(8)(p11.2q13), *NCOA3* in t(8;20)(p11.2;q12), and *EP300* in t(8;22)(p11.2;q13.2). These partner genes encode proteins that have histone acetyltransferase activity and likely contribute to leukemogenesis by altering chromatin-mediated transcriptional control of unknown target genes. The *CREBBP*(*CBP*) (16p13.3) gene is also involved in other translocations in AML—t(10;16)(q22;p13.3)/*MYST4*(*MORF*)–*CREBBP* and t(11;16)(q23;p13.3)—which results in *KMT2A–CREBBP*(*CBP*) fusion and is strongly associated with treatment-related hematologic malignancies [64].

Complex Karyotype

The definition of a complex karyotype has not yet been standardized, but the current trend is to consider patients with three or more unrelated chromosomal abnormalities (excluding those recognized as categories by WHO-2008) in this category; adults with AML who have complex karyotypes have a significantly poorer prognosis than those without a complex karyotype [65]. The loss of 17p is among the most frequent abnormalities observed as part of a complex

karyotype in hematologic disorders in adults, and it is strongly associated with abnormalities of chromosomes 5 or 7, or both [66]. The loss of 17p consistently creates a monoallelic loss of *TP53* and may be associated with *TP53* mutation or lack of expression, which is associated with a very poor prognosis; risk-adapted targeted therapies are being evaluated for patients in this category [67–69].

Cryptic Chromosomal Abnormalities and Submicroscopic Lesions

NUP98 (11p15.5)

Numerous partners have been identified for the *NUP98* (11p15.5) gene, and most partners are cryptic if the translocated genes are at the telomeric regions of the chromosomes involved. Chromosomal translocations involving *NUP98* occur in a wide range of hematopoietic malignancies. Patients harboring the cryptic t(5;11)(q35.2;p15.5)/*NUP98–NSD1* fusion usually present with a very high WBC count, have a high frequency of *FLT3*-ITD mutations, and have a dismal outcome. The overall prevalence of t(5;11)(q35.2;p15.5)/*NUP98–NSD1* is approximately 5% in children and 3% in adults with AML. Although most patients have normal cytogenetics, some can harbor +8 and del(5q) as secondary aberrations. In a large series of patients with *FLT3*-ITD, *NUP98–NSD1* was found in children (16%; median age, 10 years) and adults (8%; median age, 45 years). Among patients with normal karyotypes, *NUP98–NSD1* occurred in 8% of children and 4% of adults [70, 71]. The *NUP98–NSD1* has a distinct *HOXA/B* gene expression pattern and is associated with upregulation of other genes, including overexpression of *PRDM16(MEL1)* (mapped at 1p36.1) [72]. Of note, a cytogenetically cryptic t(11;12)(p15.5;p13.3)/*NUP98–KDM5A(JARID1A)* was recently identified in 11% of children with non-DS-AMKL (median age, 2 years). The cryptic translocation had a distinct *HOXA/B* gene overexpression signature and was associated with a poor outcome [50]. Of interest, a novel t(11;12)(p15.5;q13)/*NUP98–RARG* gene fusion was observed in a patient with AML, which resembled that seen in APL [73]. A frequent translocation in AML is the t(7;11)(p15.4;p15.5)/*NUP98–HOXA9*, with a preponderance in young adults (median age, 35 years) [74].

t(7;12)(q36.3;p13)/*MNX1–ETV6*

The t(7;12)(q36.3;p13) is seen in 20% of infants with AML, and patients harboring this translocation are younger than 2 years. The t(7;12) and *KMT2A*(*MLL*) rearrangements are mutually exclusive. The t(7;12) is a subtle chromosomal aberration and can be difficult to recognize; however, FISH with the *ETV6* probe can identify the rearrangement and detect the abnormality. Most patients with this translocation have an extra chromosome 19. The fusion predicts a poor clinical outcome in patients with AML [75, 76].

***ETV6* (12p13).** Other chromosomal abnormalities of 12p, such as deletion, balanced translocations, and unbalanced translocations, which may include the loss or rearrangement of *ETV6*, are rare in patients with AML (~3%) but have been associated with poor prognosis in children with AML [54, 77–79].

inv(16)(p13.3q24.3)/*CBFA2T3(MTG16/ETO2)–GLIS2*

The *CBFA2T3–GLIS2* fusion generated by the cryptic inv(16)(p13.3q24.3) was initially identified in 30% of children with non-DS-AMKL but not in adults. Subsequently, it was found in all morphological subtypes of AML, with an overall prevalence of 8% in pediatric AML, and in larger series, in 17% of patients with non-DS-AMKL [80, 81]. The *CBFA2T3–GLIS2* fusion occurs with normal karyotypes in 8% of children; it is rarely observed with other recurrent abnormalities such as 11q23, t(8;21)(q22;q22), or inv(16)/t(16;16)(p13.1;q22). There are very few common AML-associated mutations in these children, and the *CBFA2T3–GLIS2* fusion predicts an adverse outcome [80].

***CBFA2T3* (16q24.3)**. The *CBFA2T3* gene is also fused with *RUNX1(AML1)* in the t(16;21)(q24;q22), which is rarely identified in de novo and therapy-related AML [82]. The t(1;16)(p31;q24)/*NFIA–CBFA2T3* has been detected in young males with erythroleukemia [83].

14q32/*BCL11B*

The *BCL11B* gene is sometimes involved in mostly cryptic 14q32 translocations in AML; patients with *BCL11B* rearrangements concomitantly express myeloid and T-cell-specific biphenotypic markers and harbor *FLT3*-ITD. FISH with a break-apart *BCL11B* probe can reveal rearrangements of the 14q32 locus fused to different partner chromosomes (2q22, 6q25.3, and 8q24.21) [84, 85].

Acute Megakaryoblastic Leukemia

AMKL accounts for up to 15% of pediatric AML patients, but it is extremely rare in adults. AMKL can be divided into two subgroups: DS-AMKL and non-DS-AMKL. Non-DS-AMKL includes a heterogeneous group of patients who have poor outcomes despite receiving intensive chemotherapy

[51]. Many patients carry recurrent genetic lesions [86]. The t(1;22)(p13;q13)/*RBM15(OTT)–MKL1(MAL)* (13%) is the signature lesion for non-DS-AMKL. Other recurrent abnormalities include inv(16)(p13.3q24.3)/*CBFA2T3–GLIS2* (17 %), t(11;12)(p15.5;p13.3)/*NUP98–KDM5A(JARID1A)* (11%), and 11q23 *(KMT2A)(MLL)* (10%) [50, 80]. In general, DS-AMKL includes the majority of patients with DS who are younger than 4 years and develop AML. DS-AMKL is considered by WHO-2008 as a distinct subgroup [1]. The *GATA1* (Xp11.23) mutation is present in most patients with DS-AMKL, but it is rare in children with DS and other types of leukemias or in children with non-DS-AMKL. In patients with DS-AMKL, *GATA1* mutations confer increased sensitivity to regimens containing cytarabine. The therapy for these children is less intensive than that for AML, which results in superior outcomes (EFS > 80 %) [87, 88]. Children with DS-AMKL who are older than 4 years are usually treated on standard AML regimens and have a very poor prognosis [89].

Transient Abnormal Myelopoiesis

DS with transient myeloproliferative disorder (TMD) is observed in infants with DS and is characterized by a clonal expansion of myeloblasts that can be difficult to distinguish from that seen in AML. Most cases of TMD regress spontaneously within the first 3 months of life. TMD blasts are most commonly megakaryoblastic and have distinctive mutations involving *GATA1* (Xp11.23). TMD can occur in phenotypically normal infants with genetic mosaicism in the bone marrow for trisomy 21. Although patients with TMD rarely have other chromosomal abnormalities in addition to the +21, the presence of an aberration can predict an increased risk for developing AML [87, 90]. Approximately 20% of infants with DS and TMD eventually develop AML, most being diagnosed within the first 3 years of life [91]. This transformation likely occurs through the acquisition of additional mutations and clonal selection [86, 92].

Genetic Mutations and Cytogenetic Subgroups

Some recurrent mutations that are frequent in AML are mutually exclusive of the transcription-factor fusions generated by translocations or other structural aberrations, suggesting that a cooperating mutation such as *FLT3* might activate signal pathways important in triggering leukemogenesis. Mutations such as *NPM1*, *DNMT3A*, *IDH1,* and *TET2* are initiators of leukemogenesis. Clinically relevant mutations are usually not detected in the first years of life, but their prevalence increases with age.

FLT3 (13q12) Mutation

FLT3, a receptor tyrosine kinase expressed on hematopoietic progenitors, is frequently mutated in AML, mainly in patients with a normal karyotype and is associated with poor prognosis [93]. Mutations include internal tandem duplication (*FLT3*-ITD) and a less frequent mutation involving the region encoding the activation loop (tyrosine kinase domain, *FLT3*-TKD), leading to ligand-independent constitutive activation of FLT3 signaling. *FLT3*-ITD mutations occur in children (12%; rare in children younger than 10 years) and adults (30%) with AML. *FLT3*-TKD mutations have been identified in less than 10% of children and adults with AML, but their clinical significance is not clear [94, 95]. These mutations are particularly significant when both alleles are mutated or there is a high ratio of the mutant allele to the normal allele [96, 97]. Approximately 10% of *FLT3* mutations are either gained or lost at relapse, which suggests that they can be late subclonal events and are not always the driver mutation of prognosis and response [98]. *FLT3*-ITD mutations are particularly prevalent in APL (up to 40% of children and adults), but their prognostic impact is minimal [24]. There is also a strong association of *FLT3*-ITD mutations with t(6;9)(p23;q34)/*DEK–NUP214* (~70%) and t(5;11)(q35;p15.5)/*NUP98–NSD1* (~82%). Both abnormalities are associated with dismal outcome, but this is independent of the presence of *FLT3*-ITD [57, 71].

Isolated trisomy 13 is extremely rare in children, but has been reported in a low incidence in adults with AML or MDS. Such patients have a poor outcome; however, recent reports have shown that these patients may be sensitive to lenalidomide as are patients with low-risk MDS with the 5q- cytogenetic abnormality [99]. Trisomy 13 (chromosome 13 harbors *FLT3*) is strongly associated with *RUNX1*(*AML1*) mutations and increased *FLT3* expression in AML.

NPM1 (Nucleophosmin) (5q31) Mutation

The NPM1 protein has diverse functions in the cell, such as chromatin remodeling and ribosomal complex assembly. *NPM1* mutations are detected by immunohistochemical methods that show its cytoplasmic localization [100]. NPM1 mutations are found in up to 35% of adults and 8% of children with AML. These mutations are most frequent in cytogenetically normal AML (45–60% of patients), but can be associated with +4, +8, and del(9q) and additional gene mutations such as *FLT3*-ITD, *DNMT3*, *IDH1,* and *IDH2* [101]. *NPM1* mutations are a good marker to assess MRD [102]. *NPM1* mutations are associated with good prognosis if *FLT3*-ITD or *DNMT3* mutations are absent [103].

CEBPA (19q13.1) Mutation

CEBPA encoding a transcription factor involved in normal myelopoiesis is mutated in 10% of adults younger than 60 years and in 5% of children with AML [57]. *CEBPA* mutations are found in approximately 10% of patients with normal karyotypes and can occur in patients with abnormal karyotypes, but are mutually exclusive of recurrent chromosomal aberrations [104]. Reports suggest that only biallelic mutations of *CEBPA* associated with a normal karyotype predict a favorable outcome in the absence of *FLT3*-ITD mutations [105–107].

Summary

Conventional and molecular cytogenetics are among the most important features currently used to direct treatment randomization in AML. In the last 30 years, many recurrent genetic lesions have been identified, but no single mutation has been shown to be sufficient to cause leukemogenesis. Instead, cooperating mutations are believed to promote proliferation and survival of aberrant cells or block the differentiation necessary for leukemic transformation. Most of the recurrent chromosomal abnormalities identified in myeloid blast cells generate chimeric oncoproteins that inhibit differentiation. Recent molecular genetic studies have shown that the common molecular markers for subclasses of AML include genetic lesions that may or may not be associated with chromosome abnormalities as determined by conventional cytogenetics [108–110]. The advent of novel technologies available for the genomic sequencing of leukemic blasts will help enhance our understanding of the pathophysiology of acute leukemias and identify novel therapeutic targets. Collaborative trials are needed given the rarity of childhood AML, its biological heterogeneity, and the prospect of targeted therapeutics for small genetic subgroups [10, 111].

Acute Lymphoblastic Leukemia

ALL is the most common leukemia in children, where 80–85% of leukemias are ALL, and is much rarer in adults where only 20% of acute leukemia is ALL. The cytogenetics of ALL has long been known to be of prognostic relevance, and both the number of chromosomes (modal number) and some structural abnormalities are prognostic in both adult and pediatric ALL [112].

Both cytogenetic aberrations and outcome differ between children and adults with ALL. Children with ALL have a very good prognosis, with 5-year EFS rates approaching 90% [113]. On the other hand, adults with ALL have a much poorer outcome with an overall EFS of 40% [114]. This difference in outcome can in part be attributed to the different biology of ALL in the two age groups, some of which is demonstrated by cytogenetics. The frequencies of "good prognosis" cytogenetic aberrations are higher in children than in adults with ALL, and "poor prognosis" cytogenetic abnormalities are more frequent in adults than in children with ALL.

ALL also is differentiated by the cell type that becomes leukemic. Approximately 85% of children and 75–80% of adults with ALL have B-cell precursor (BCP) ALL, and the remaining have T-cell ALL (T-ALL) [115, 116]. The cytogenetics of BCP and T-ALL differ, although there are some overlaps, and will be discussed separately. (See Table 16.2 for summary of ALL cytogenetic aberrations.)

B-Cell Precursor ALL

Chromosome Numbers in ALL

Modal number (mn) is defined as the number of chromosomes in the most prevalent abnormal clone. However, in ALL, the mn for classification is considered the number of chromosomes in the simplest abnormal clone, regardless of the prevalence of the clone. The mn is prognostic in ALL. Modal number classification in ALL includes high hyperdiploidy (HH, 51–67 chromosomes), low hyperdiploidy (LH, 47–50 chromosomes), near-triploidy (68–79 chromosomes), near-tetraploidy (>79 chromosomes), pseudodiploidy (46 chromosomes with an abnormality, usually, although not always, structural), normal chromosomes, and hypodiploidy (HO, <46 chromosomes).

As per mn classification, patients with HH have the best outcome. About 35% of children with ALL have HH [unpublished Children's Oncology Group (COG) data], whereas <10% of adults have HH [117, 118]. Chromosomes X, 4, 6, 10, 14, 17, 18, and 21 are preferentially present in extra copy number in HH ALL [119, 120] (Fig. 16.3). Furthermore, the excellent prognosis of HH ALL can be attributed to the presence of specific extra chromosomes. The COG found that the simultaneous presence of extra copies of chromosomes 4, 10, and 17 was associated with an excellent prognosis [121], and the Medical Research Council (MRC) of the United Kingdom found that extra copies of chromosomes 10 and 18 were associated with an excellent prognosis [119]. The significance of specific extra chromosomes has not been studied in adults with ALL. Approximately 50% of children with HH ALL have a structural abnormality, most often a duplication of 1q, which does not have prognostic significance [122]. The only other recurring structural abnormality frequently found in pediatric HH ALL is a t(1;19)(q23;p13.3), which frequently does not have a *TCF3-PBX1* rearrangement. Adults with HH ALL, however, frequently have recurring structural abnormalities that are associated with an adverse outcome [118].

Table 16.2 Common recurring aberrations in ALL

B-cell precursor ALL						
	Frequency (%)					
Aberration	Adult	Pediatric	Genes	Prognosis	Cytogenetic variants	Comments
Numerical aberrations						
Hyperdiploidy, mn > 50	<10	25–30		Good		
Hyperdiploidy, mn > 46 < 51	15	15		Intermediate		Usually have structural abnormalities, ~50% recurrent
Pseudodiploidy				Intermediate		
Near-triploidy and mn 30–39	Rare			Poor		
Hypodiploidy, mn 44–45		7		Intermediate		Usually have structural abnormalities
Hypodiploidy, mn <44		1.7		Poor		Few structural abnormalities, frequent doubling in mn <40
Structural aberrations						
t(9;22)(q34.1;q11.2)	25	<4	*BCR-ABL1*	Intermediate with TKI therapy	5% complex	Frequency increases with age
t(12;21)(p13;q22)	Very rare	25	*ETV6-RUNX1*	Good		Cytogenetically cryptic. Many other *ETV6* rearrangements may differ in clinical characteristics and prognosis
t(4;11)(q21.3;q23)			*KMT2A-AFF1*	Poor	Insertions, three-way translocations	Frequent in infants. Outcome may differ with age
t(11;19)(q23;p13.3)			*KMT2A-MLLT1*	Poor		Also in T-ALL and AML
t(9;11)(p22;q23)			*KMT2A-MLLT3*	Adults better than other 11q23; children poor		
Other 11q23	5	5 >1 year 80 <1 year	*KMT2A/other*	Poor		Frequent in infants. Outcome may differ with age and partner
t(1;19)(q23.3;p13.3)	3	6	*TCF3-PBX1*	Not prognostic	der(19)t(1;19)(q23.3;p13.3); t(17;19)(q22;p13.3)	*HLF-E2A* in t(17;19)—poor prognosis
der(21)/add(21)			*RUNX1*	Poor	der(21) appears in many guises	Amplification of *RUNX1* or near gene
del(9p)	30	6	*CDKN2A*	Unknown		
dic(9;12)(p13;p11.2)	Rare	Rare	50% *PAX5-ETV6*	Good		Not all have PAX5/ETV6 rearrangement
dic(9;20)(p12-p13;q11.2)	Rare	Rare	*Some PAX5-ASXL1*	Good		
dic(7;9)(p11.2;p13)	Rare	Rare				
t(8;14)(q24;q32.3)			*IGH-MYC*	Intermediate		Usually Burkitt leukemia/lymphoma
t(8;14)(q11.2;q32.3)			*IGH-CEBPD*	Good in Down syndrome		Prevalent in Down syndrome ALL
t(5;14)(q31;q32.3)	Rare	Rare	*IGH-IL3*	Poor		Associated with hypereosinophilia
Other 14q32.3			*IGH*			
del(6q)	7	1.5	*MYB* some cases	Not prognostic		Frequent in *ETV6-RUNX1* cases
del(5q)	Rare	Rare				
del(7p)	4	4		Poor		
del(12)(p11.2-p13)			*?ETV6*	Not prognostic		
Sole +8	3	3		Unknown		

(continued)

Table 16.2 (continued)

B-cell precursor ALL						
	Frequency (%)					
Aberration	Adult	Pediatric	Genes	Prognosis	Cytogenetic variants	Comments
Sole +X	Rare	Rare		Unknown		
Sole +21	Rare	Rare				
del(13q)monosomy 13	5	2		Unknown		
T-lineage ALL[a]						
14q11.2 rearrangement	35		*TRA/TRD*	Not prognostic	7p14, 7q34	*TRG,TRB*
t(1;14)(p32;q11.2)		10 translocations 30 deletions	*TRA/TRD-TAL1*	Favorable	del(1)(p32p32) cryptic	*TAL1-STIL*
t(11;14)(p13;q11.2)	5–10		*TRA/ TRD-LMO2*			
t(11;14)(p15;q11.2)	2		*TRA/ TRD-LMO1*			
t(10;14)(q24.32;q11.2)	5–10		*TRA/TRD-TLX1*	Favorable		
inv(7)(p15.2q34)	3–5		*TRB-HOXA*			
t(6;7)(q23;q34)	3		*TRA/TRD-MYB*			
t(6;11)(q27;q23)	Rare		*KMT2A-MLLT4*	Poor		Also in AML
t(11;19)(q23;p13.3)			*KMT2A-MLLT1*	Favorable		
ins(10;11)(p12;q23q13)			*KMT2A-MLLT10*	Poor		May be cryptic
t(10;11)(p12;q14)	2–3		*PICALM-MLLT10*	Poor		Also in AML
t(5;14)(q35.1;q32)	13	20	*TLX3-BCL11B*	Poor	*TLX3* rearranges with other partners	Cryptic
amp(*NUP421-ABL1*)	<6		*NUP214-ABL1*	Poor		Cryptic, FISH required, rearrangement on an episome
del(9)(p13-p22)	65		*CDKN2A-CDKN2B*			Frequently cryptic
del(6)	10–20		*?MYB*			

[a]When frequencies do not differ between children and adults, they are displayed in the adults column

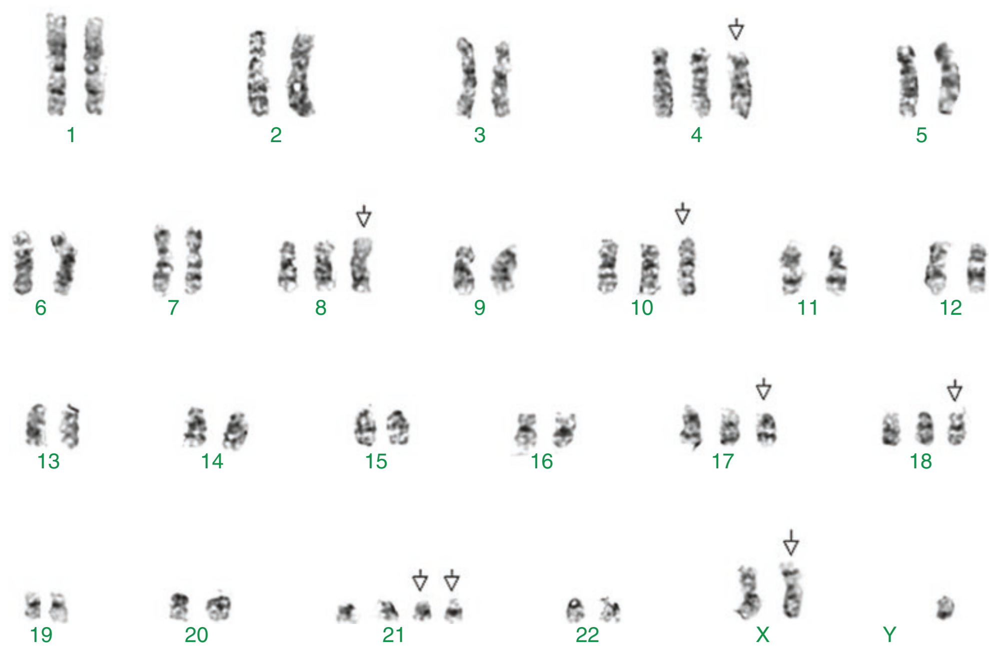

Fig. 16.3 Karyogram from a child with high hyperdiploid ALL. *Arrows* indicate extra chromosomes. The karyotype for this patient is 54, XY, +X, +4, +8, +10, +17, +18, +21, +21

Low hyperdiploidy is present in approximately 15% of both pediatric and adult ALL. These patients frequently have a recurring structural abnormality, and prognosis is associated with the recurring structural abnormality. The remaining patients with LH ALL have an intermediate prognosis [123, 124].

Near-tetraploidy is rare, occurring in <1% of pediatric and adult BCP ALL. It is more frequent in T-ALL. In pediatric BCP ALL, near-tetraploidy often also has an *ETV6-RUNX1*(*TEL-AML1*) rearrangement, which is associated with excellent prognosis (see in text to come). Because near-tetraploidy is rare, its prognostic significance in the absence of an *EVT6-RUNX1* rearrangement is unknown [117, 118].

Near-triploidy is also rare (<1% of pediatric ALL and 4% of adult ALL). These cases appear to be "true" near-triploids in pediatric ALL, with three copies of most chromosomes and only rarely structural abnormalities. Adult near-triploidy appears to be associated with hypodiploidy and represents a doubling of a hypodiploid clone. Cases with this karyotypic result are classified as hypodiploid cases in pediatric ALL. The prognosis of "true" near-triploidy is not known.

Pediatric ALL with hypodiploidy is further differentiated by mn. Seven percent of children with BCP ALL have 44 or 45 chromosomes. These cases usually have a structural abnormality, and prognostic significance is associated with the structural abnormality. Overall, these patients have an intermediate prognosis [125]. Patients with 43 and fewer chromosomes (1.7% of pediatric BCP ALL) have a dismal prognosis [125]. Cytogenetically, they can be classified as those with 40–43 chromosomes, with 30–39 chromosomes, or with 24–29 chromosomes [126, 127]. Patients with 40–43 chromosomes nearly always have a structural abnormality (>86%), and do not have doubling of the abnormal clone. Karyotypes of children with 30–39 chromosomes have doubling in 30–57% of cases and often (39–57% of cases) have structural abnormalities. Patients with 24–29 chromosomes (near-haploidy, 1% of pediatric BCP ALL) frequently also have doubling of the near-haploid clone (57–64% of cases), and structural abnormalities are found in only 14–25% of cases [126, 127]. Doubling of the hypodiploid clone can be masked, with only the doubled clone present. In such cases, additional studies, such as SNP microarray, are required to detect the abnormal clone and assure correct cytogenetic classification. Recent molecular studies of pediatric hypodiploid ALL have confirmed three categories of hypodiploidy differing in both modal number and types of mutations in the different groups [128]. Near-haploid cases (mn = 24–31) frequently have mutations in the signaling pathway, particularly in *NRAS*, *NF1*, and *IKZF3*. They also tend to be younger than hypodiploid patients with higher mn. Low hypodiploid cases (mn = 32–39) have mutations in *RB1* and *IKZF2* and significantly in *TP53*. Approximately half of the latter are germ line in origin, consistent with Li-Fraumeni syndrome. Adults with ALL with near-triploidy and hypodiploidy (30–39 chromosomes) are often classified together as the near-triploid clones usually represent doubling of the hypodiploid clone [117, 118]. Near-haploid adult ALL is extremely rare, and has not been reported in patients older than 40 years [117].

Recurring Structural Abnormalities in BCP ALL

Cytogenetic classification of BCP ALL is further classified on the basis of structural abnormalities. In cases with a recurring structural abnormality and a numerical abnormality, the case is generally classified by the structural abnormality.

Rearrangements Associated with Favorable Prognosis

The most common structural abnormality in pediatric BCP ALL is t(12;21)(p13;q22), which results in an *ETV6-RUNX1*(*TEL-AML1*) fusion gene. The abnormality is cryptic by standard cytogenetics, but can be detected by FISH using probes for *ETV6-RUNX1*. This rearrangement is present in 25% of children with BCP ALL and is associated with excellent prognosis [129]. It is rarely found in adult ALL, and then with rare exception, in patients younger than 30 years [130]. Although the t(12;21) is cryptic, 80% of these patients have cytogenetically visible aberrations [131]. The *ETV6-RUNX1* rearrangement is prenatal in origin [132]. It is not sufficient to cause leukemia, but appears to be an initiating event. A second event is required for expression of the disease [132]. The second event is often loss of the second *ETV6* allele, which is frequently detected by standard cytogenetics. This may explain early studies reporting either no prognostic significance or a good prognosis for patients with deletions of 12p.

Rearrangements Associated with Adverse Prognosis

Rearrangements of *KMT2A* (formerly *MLL,* 11q23) are associated with a poor prognosis in ALL. Although *KMT2A* rearrangements occur in both ALL and AML, some rearrangements are more common to each leukemia. *KMT2A* rearrangements are present in 2–7% of ALL patients older than 12 months. However, they are particularly common in infants, and up to 80% of infants younger than 12 months who are diagnosed with ALL have an *KMT2A* rearrangement. A t(4;11)(q21;q23)/*KMT2A-AFF1*(*AF4*) occurs in ALL, but the cells may have some myeloid markers [133]

(Fig. 16.4). It is the most common *KMT2A* rearrangement in infant ALL and is associated with a very poor prognosis [134]. The t(4;11) also occurs in pediatric and adult ALL, although at a much lower frequency (<2%), and also is associated with a poor prognosis. The etiology of the t(4;11) in infants and young children is prenatal [132]. Studies of monozygous twins with t(4;11) ALL show that they share identical molecular rearrangements, and studies of infant blood spots (Guthrie spots) from children with t(4;11) ALL show that these rearrangements can be detected in blood spots [132]. Also, cord blood investigations have shown that the rearrangement is much more prevalent than the development of leukemia [132]. These studies indicate that additional mutations are required for the development of ALL; even though a t(4;11) may occur prenatally, t(4;11) leukemia does not always develop, and when it does develop there is typically a time lag. The second most frequent *KMT2A* rearrangement in infant ALL is t(11;19)(q23;p13.3)/*KMT2A-MLLT1*(*ENL*), which also portends a poor prognosis, although it may not be as poor as t(4;11) infant ALL (Fig. 16.2). This rearrangement occurs in 3–7% of children and adults with ALL as well as in AML. A third common *KMT2A* rearrangement in ALL that occurs in all age groups is t(9;11)(p22;q23)/*KMT2A-MLLT3*(*AF9*), which is also very common in AML. Other *KMT2A* partners in ALL include Xq13.1/*FOXO4* (*AFX1*), 1p32/*EPS15*(*AF1P*), 5q31/*AFF4*(*AF5q31*), 6q27/*MLLT4*(*AF6*), 7p22.1/ *TNRC18*(*KIAA18856*), 10p12/*MLLT10*(*AF10*), 11q21 /*MAML2*, 11q23.3/*BCL9L*, 15q14/*CASC5*(*AF15Q14*), 16p13.3/*CREBBP*(*CBP*), 19p13.3/ *ASAH3*(*ACER1*), 19q13/ (*ACTN4*), 20q11/*MAPRE1*, and 22q11.21/*DEPT5*(*CDCREL*) [6].

Secondary ALL is a rare event, and most frequently has an *KMT2A* rearrangement. These secondary ALL generally occur after a patient has been treated with epipodophyllotoxins; they have a short lag time of 2–4 years and no preceding preleukemic phase. Patients with secondary ALL typically respond to therapy, but remissions are short and prognosis is poor. A t(4;11) is the most common *KMT2A* rearrangement in therapy-related ALL, but other *KMT2A* rearrangements, particularly t(9;11), also occur [6].

Philadelphia Chromosome (Ph+) and Philadelphia-like ALL

A Philadelphia chromosome (Ph), resulting from a t(9;22)(q34.1;q11.2) and a *BCR-ABL1* fusion, in ALL was associated with a very poor outcome in the past [114, 135] (Fig. 16.5). However, the poor outcome has been ameliorated by treatment with the tyrosine kinase inhibitor, imatinib mesylate [136, 137]. This translocation is present in less than 4% of children with ALL, but in 25% of adults with ALL. Its frequency increases with age, at least up to the fourth decade [117].

Philadelphia-like or *BCR-ABL1*-like ALL has an expression signature very much like that of Ph+ ALL, but without a t(9;22)(q34.1q11.2) [138, 139]. It is 3–4 times more common than Ph+ ALL in children, and its frequency increases with age. Outcome with traditional treatments is very poor. It is associated with *IKZF1* deletion, *CRLF2* rearrangements, and *JAK2* mutations. There are two different types of Ph-like ALL: those associated with *CRLF2* overexpression [140] and those with a variety of gene fusions targeting kinase

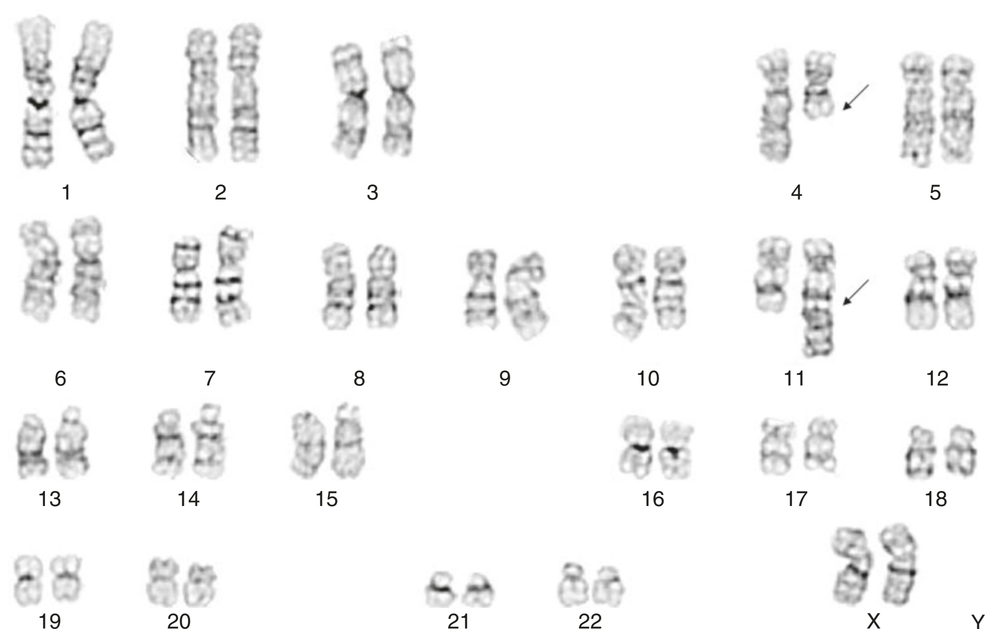

Fig. 16.4 Karyogram of patient with ALL and a t(4;11). *Arrows* indicate the abnormal chromosomes. The karyotype for this patient is 46,XX,t(4;11)(q21;q23)

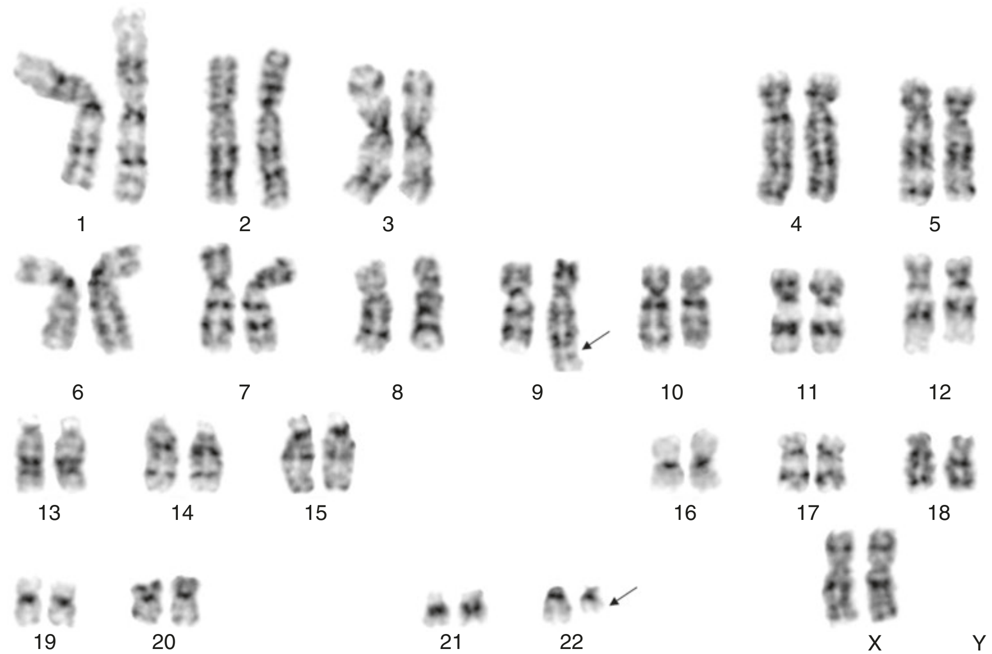

Fig. 16.5 Karyogram from a Philadelphia chromosome-positive ALL patient. *Arrows* indicate the abnormal chromosomes. The karyotype for this patient is 46,XX,t(9;22)(q34.1;q11.2)

genes, including *ABL1*, *ABL2*, *CSF1R*, *PDGFRB*, *EPOR*, and *JAK2* in a significant percentage of the remaining cases. Patients with these aberrations may be sensitive to tyrosine kinase inhibitors [141, 142]. Many of these gene fusions are cytogenetically cryptic, and molecular techniques are required to detect them. *CRLF2* is located in the pseudo-autosomal region of the X and Y chromosomes. CRLF2 overexpression results from a translocation with *IGH* or from a deletion of the sequences between *CRLF2* and *P2RY8*; both are cytogenically cryptic, and FISH or other molecular techniques are required for their detection. The *P2RY8-CRLF2* deletion is particularly common in children with DS [143]. Patients with a *CRLF2* rearrangement may be sensitive to ruxolitinib [142].

In addition to a t(12;21), the *ETV6-RUNX1* probes detect amplification of the *RUNX1* gene, resulting in an abnormality designated *iAMP21*. This may be detected with array studies, but is typically detected by FISH. By definition, amplification requires at least three extra copies of *RUNX1* on a single chromosome, usually an abnormal chromosome 21, resulting in a total of at least five *RUNX1* signals. It is not known whether the significant gene amplified in this region is *RUNX1*, as *RUNX1* is not overexpressed, although aCGH studies have shown that *RUNX1* is always included in the amplified region. The abnormality has been associated with a poor outcome [144, 145]. This aberration is more common in older children, but is rarely seen in adults with ALL [146].

Rearrangements Associated with Uncertain Prognostic Significance

A t(1;19)(q23.3;p13.3)/*TCF3*(*E2A*)-*PBX1* occurs both as a balanced t(1;19) and an unbalanced der(19)t(1;19) with two normal chromosomes 1. It is found in 6% of pediatric and 3% of adult ALL, typically with a pre-B-cell phenotype. It is only rarely prenatal in origin [132]. Early reports indicated an adverse prognosis for children with this aberration, but subsequent studies showed that it did not influence outcome, most likely due to more intense treatment in subsequent studies [130]. However, a t(1;19) may portend an adverse prognosis in adult ALL [147].

Deletions of 9p are frequent in ALL, occurring in up to 30% of adult ALL and up to 6% of pediatric ALL. Some are seen by traditional cytogenetics, but many are cryptic and cannot be detected by cytogenetics, so the frequency of this aberration may be much higher. The clinical significance of this abnormality has been controversial, with some studies showing an adverse prognosis [148] and others no impact on outcome [149]. The abnormality is thought to result in loss of the oncogene *CDKN2A*, which can be detected by FISH.

Interestingly, dicentric chromosomes are frequent in ALL, and they most often involve a chromosome 9. Although all dic(9;V) result in loss of 9p, the significance of dic(9;V) may be associated with a fusion gene rather than a deletion of 9p. A dic(9;12) is one of the more frequent dicentric chromosomes in ALL, although it is seen in less than 1% of

children with ALL and very rarely in adults with ALL (usually in young adults) [150]. Approximately 50% of cases with dic(9;12) have a *PAX5-ETV6* rearrangement [151], and the remaining have an *ETV6-RUNX1* rearrangement instead [152]. The chromosome 12 involved in the dic(9;12) is not involved in the *ETV6-RUNX1* rearrangement, and the genes involved in the dic(9;12) in these cases have not yet been identified [152]. There is no consensus on the outcome of patients with dic(9;12); early reports of dic(9;12) indicated an excellent outcome [150], but studies by the Children's Cancer Group did not confirm this good prognosis [148].

A dic(9;20)(p12–p13;q11.2) is another frequent dicentric chromosome in ALL. This aberration is subtle and may be missed by traditional cytogenetics; cases with monosomy 20 need to be tested for the presence of this dicentric chromosome by FISH with probes for the centromeres of chromosomes 9 and 20 on either metaphases or interphases or using chromosome paints for chromosomes 9 and 20 on metaphases. The dic(9;20) has a peak incidence at age approximately 3 years and has a female predominance. It occurs in approximately 0.5% of adults and 2% of children with ALL and appears to have a favorable prognosis [153]. The molecular breakpoints of both partners of the dic(9;20) are heterogeneous—some 9p11–13 breakpoints occur in the repetitive region near the centromere and some involve *PAX5*. The 20q breakpoint frequently involves *ASXL1* [154].

Rearrangements of 14q32, usually involving the *IGH* gene, are recurrent in ALL. The t(8;14)(q22;q32.3)/*IGH-MYC* and its variants t(2;8)(p12;q22)/*IGK-MYC* and t(8;22)(q22;q11.2)/*IGL-MYC* generally indicate Burkitt leukemia. Although this aberration can occur in patients with BCP ALL, most are considered mature B-cell leukemias. Several rearrangements of 14q32.3, in addition to t(8;14)(q24;q32.3), are recurrent in ALL. They result in overexpression of the *IGH* partner gene. One such rearrangement is a t(8;14)(q11.2;q32.3)/*IGH-CEBPD*, which is more frequent in children with DS and ALL. In non-DS cases, trisomy 21 is often a secondary abnormality. Patients with DS and ALL who have this abnormality have an excellent prognosis [155]. Another *IGH* rearrangement in ALL is t(14;19)(q32.3;q13)/*IGH-CEBPA*. Additional *CEBP* genes also rearrange with *IGH*, indicating that overexpression of any of the several members of a gene family can contribute to ALL [156].

Recurrent Deletions and Single-Chromosome Aneusomies

Deletions of 12p are frequent in ALL. A del(12p) has been detected by cytogenetics in 9% of children and 4% of adults with ALL [118, 157]. It often includes deletion of *ETV6* and is sometimes associated with t(12;21) in pediatric ALL. When examined regardless of the presence of t(12;21), it has no prognostic significance [157]. Its clinical significance in the absence of t(12;21) remains to be investigated.

Deletion 6q is more frequent in adults (7%) than in children (1.5%) with ALL ([124] and COG unpublished data). It is often cryptic, and therefore its true incidence is not known. Whether the *MYB* oncogene is the significant gene deleted is controversial. When detected by traditional cytogenetics, del(6q) does not have prognostic significance in pediatric ALL [158], although it may have an adverse impact on prognosis in adult ALL [117, 159].

Deletions of 5q in ALL are rare (<2% of both children and adults). They do not appear to have prognostic significance in adults [160]. Children with ALL and a del(5q) have an adverse EFS, but not overall survival, indicating that they may respond to salvage therapy [161]. Whether the significant region of 5q lost in ALL is the same as that in AML remains to be investigated.

Monosomy 7 is rare but recurrent in ALL. It is frequently a secondary abnormality in Ph+ ALL. Deletions of chromosome 7 also occur, most frequently of 7p. When Ph+ cases are excluded, monosomy 7 or chromosome 7 deletions occur in approximately 5% of adults and children with ALL. Deletions of 7p appear to be an adverse prognostic factor in children but not adults with ALL [118, 162].

Trisomy 8 as a sole abnormality is also rare but recurrent in ALL, seen in approximately 3% of cases ([124] and COG unpublished data). There is no agreement on its prognostic significance: some studies have shown no prognostic significance [118], and others have shown an adverse outcome for patients with ALL and this abnormality [159].

Gain of an X chromosome in non-HH ALL is also recurrent, often in conjunction with another abnormality, such as the t(8;14)(q11.2;q32.3). As a sole cytogenetic abnormality, it is found in less than 1% of children with ALL (unpublished COG data), and its frequency in adults as a sole abnormality has not been reported. It does not appear to influence prognosis in adults when all cases with an extra X chromosome are considered [118].

Trisomy 21 in non-HH ALL is also recurrent. As with an extra X chromosome, it often occurs in conjunction with another abnormality, such as the t(12;21), wherein it is a frequent secondary abnormality. Sole trisomy 21 is rare in pediatric ALL, occurring in less than 1% of cases (unpublished COG data). Its frequency in adult ALL or its prognostic significance as a sole aberration in ALL has not been investigated.

Deletions of 13q and monosomy 13 occur in 5% of adults with ALL, and 2% of children with ALL have breakpoints in 13q12–14 [163]. In children, these aberrations contribute to a higher risk of treatment failure and are also associated with other adverse clinical features. Deletion 13/monosomy 13 does not appear to have prognostic significance in adults [118].

B-Cell Precursor ALL Summary

Frequencies of cytogenetic aberrations known to have the most significant impact on outcome differ in children and adults with ALL. The differences in frequencies of a Ph, an *ETV6-RUNX1* rearrangement, and HH contribute to the differences in outcome between pediatric and adult ALL. HH and an *ETV6-RUNX1* rearrangement, both rare in adults, have the best prognosis among all ALL cytogenetic aberrations. However, a Ph, which is much more common in adults than in children with ALL, predicts a poor prognosis, especially before the advent of treatment with tyrosine kinase inhibitors specific for this rearrangement. The frequencies of aberrations with less prognostic impact differ only slightly between adults and children with BCP ALL. Additional research is needed to elucidate the etiology of the differences in frequencies of ALL cytogenetic subsets between adults and children with ALL. Although the genes involved in many aberrations are known, many have not been identified. Identification of new aberrations, such as Ph-like ALL and development of treatments specific for genetic subsets of ALL will contribute to improved outcomes for these patients in the future.

T-Cell ALL

T-ALL is less common than BCP ALL, accounting for 10–15% of pediatric ALL and 25% of adult ALL, and is more frequent in males than in females [116]. It is cytogenetically distinct from BCP ALL, although both subtypes have some aberrations in common. It is typically subdivided according to the maturation status of the leukemic cells. Normal metaphase cytogenetics in T-ALL may in part be due to a high frequency of cryptic aberrations in T-ALL. Molecular approaches have shown that 90% of adults with T-ALL have aberrations [164]. Numeric changes are rare in pediatric T-ALL (<8%; unpublished COG data), but are more common in adult ALL (~30%) [118].

The most common cytogenetic aberrations in T-ALL involve the T-cell receptor (TCR) loci, *TRA/TRD* at 14q11.2, *TRB* at 7q34, and *TRG* at 7p14, and occur in 35% of cases of T-ALL [165]. Any of the TCRs can rearrange with each of the partner loci, and some partners appear to preferentially rearrange with different TCRs. As a result of these rearrangements, the translocation partner of the TCR is juxtaposed to the promoter or enhancer element of the TCR and is overexpressed. A t(1;14)(p32;q11.2)/*TRA/TRD-TAL1* results in overexpression of *TAL1*. This aberration is rare (3%) in T-ALL. However, a cryptic deletion of 1p, which results in loss of *SCL* and juxtaposition of *STIL* and *TAL1*, is much more common (17% of T-ALL) and also causes deregulation of *TAL1*. This rearrangement is associated with a good prognosis [166]. Other recurring translocations with the TCR loci are with 10q24/*TLX1*(*HOX11*), 9q34.3/*NOTCH1*, 11p13/*LMO2*, 11p15/*LMO1*, 7p15/*HOXA* cluster, 9q32/*TAL2*, 8q24/*MYC*, 19p13/*LYL1*, 1p34/*LCK*, 14q13/*NKX2.1*, and 12p13/*CCND2*. *TLX1* expression is generally associated with an improved outcome, and its expression also occurs in the absence of a *TLX1* rearrangement [167]. The prognostic significance of the other genes rearranged with TCR loci is not well established. The recurrent t(7;9)(q34;q34.3) led to the identification of Notch1 signaling in T-ALL pathogenesis, and *NOTCH1* activating mutations are now known to occur in 60% of T-ALL [168, 169]. Notch1 is important in regulating hematopoietic progenitor commitment to the T-cell lineage, consistent with *NOTCH1* mutations in 50–70% of T-ALL [170, 171].

Chimeric or fusion genes also recur in T-ALL, including *KMT2A* rearrangements, although these are rare (<5%; COG unpublished data and [116]). Interestingly, a t(11;19)(q23;p13.3) (*KMT2A-MLLT1*) rearrangement in T-ALL is associated with a relatively good prognosis [172] (Fig. 16.2). A t(10;11)(p12;q14)/*PICALM*(*CALM*)-*MLLT10* occurs in 2–3% of patients with T-ALL. It can be mistaken for a *KMT2A* rearrangement, as the breakpoints of different t(10;11) can be difficult to distinguish. These abnormalities can be detected by FISH. Patients with a *PICALM-MLLT10* fusion have a relatively good prognosis [116]. A t(5;14)(q35;q32)/*BCL11B-TLX3* is another cryptic rearrangement in T-ALL. *TLX3* also rearranges with other partners, including 14q11.2/*TRA/TRD*), 7q21/*CDK6*, and 5q34/*RANBP17*. As a result of these rearrangements, *TLX3* is overexpressed. The rearrangements are rare and previously appeared to predict a poor prognosis; however, with current therapies these patients may not have a poor prognosis [116, 173]. In most cases with *TLX3* expression, a chromosome 5 abnormality is present [173]. Amplification of a cryptic rearrangement of *NUP214-ABL1* is seen in 2–6% of patients with T-ALL. *NUP214* is slightly distal to *ABL1* on chromosome 9 band q34. This rearrangement is unique in that it is present as an episome (a small extrachromosomal circular body) with juxtaposition of these genes. The episomes are in the nucleoplasm. Although not detected by metaphase cytogenetics, this rearrangement can be detected by FISH with either a fusion probe for *NUP214-ABL1* or a probe for *ABL1* [174]. This aberration is also found in Ph-like B-precursor ALL [141]. There is some preliminary evidence that patients with this aberration may respond to imatinib therapy [175].

Deletions of 9p are very frequent in T-ALL; they are often cryptic and not detected by cytogenetics, and therefore FISH or PCR is needed for their detection. The genes deleted are *CDKN2A*, the primary target, and *CDKN2B*, also very frequently deleted. They are often homozygously deleted (65% of T-ALL) and less frequently hemizygously deleted (23%) [176]. In addition, they may be inactivated by methylation;

up to 93% of *CDKN2A* and 99% of *CDKN2B* may be inactivated at the protein level [177]. This aberration appears to have no prognostic significance [116]. Similarly, deletions of 6q are common, occurring in 30% of cases, but their prognostic significance is uncertain.

Although complexity has not been shown to be prognostic in BCP ALL, it may be prognostic in T-ALL, particularly in adults; 5–8% of adults with T-ALL have five or more abnormalities, and 15% have three or more abnormalities. In a large study, adults with T-ALL who had five or more abnormalities had a poor outcome [116].

T-ALL Summary

In normal development, T-cell progenitors originate in the bone marrow and migrate to the thymus, where they complete antigen-independent maturation. The thymus is encapsulated and has cortical and medullary areas. T-cell precursors enter the subcapsular cortical area where they proliferate and begin to differentiate. As the cells differentiate, they move from the cortex toward the medulla where differentiation continues, TCR rearrangement begins, and cells progress through differentiation stages; first expressing neither CD4 nor CD8, then expressing CD4 and CD8, and then either CD4 or CD8. The cells move to the cortico-medullary junction and maturation continues. When the cells are mature, they leave the thymus and enter the periphery.

Expression array studies of T-ALL have shown five different expression signatures, which correspond to differentiation stages and to cytogenetic aberrations [178]. The *TAL1/LMO* subgroup includes all rearrangements involving these genes (Table 16.2) and the T-cells are in an early stage of development, when neither CD4 nor CD8 is expressed. The *TLX1* subgroup includes cases with *TLX1* (10q24) rearrangements, typically affects cells at the early cortical thymocyte stage, and is associated with a good prognosis. The *TLX3* subgroup currently includes only *TLX3* (5q35) rearrangements. Although they do not have a unique differentiation profile, they do not have a mature immunophenotype. The fourth expression array subgroup is the *HOXA* subgroup and includes *PICALM-MLLT10*, *KMT2A*, inversion 7, and *SET-NUP214* rearrangements. *HOXA* genes are activated in this subgroup, and they generally have a mature immunophenotype. The *MYB* subgroup represents cases with expression of *MYB* through activation by a translocation with a TCR or by *MYB* amplification. The T-cell developmental stage of this subgroup has not been identified [178]. These expression array studies and their correlations with cytogenetic aberrations show a pattern of genetic lesions in T-ALL and a relationship to the differentiation status of the transformed T-cells. The different patterns of gene expression categorize many recurring T-ALL cytogenetic aberrations into groups, illustrating association of different abnormalities to each other, to T-cell differentiation, and to prognosis.

Summary

The use of conventional and molecular cytogenetics has led to many gene discoveries in cancer biology over the past several decades. The cloning of chromosome translocation breakpoints, like the t(8;21), inv(16), 11q23, t(12;21), t(9;22), and many others, has helped to identify the genes involved in these translocations and allowed the development of DNA probes for FISH and RT-PCR that are used to diagnose and monitor minimal residual disease. Many genetic lesions are currently used as tools for the diagnosis, classification, and management of patients with acute leukemia.

The advent of new DNA array and sequencing technologies has enabled the simultaneous detection of mutations, deletions, amplifications, and epigenetic changes; and RNA arrays have allowed the evaluation of associated biochemical pathways. Thus, the detection of additional tumor markers via innovative genetic technologies will help pinpoint the genes that act cooperatively in a particular malignant cell type, complementing the current WHO-2008 assignment of genetic lesions used in acute leukemias.

Currently known molecular genetic mutations as well as novel ones that remain to be identified are potential genetic markers that have clinical significance, underscoring the patient variability in and heterogeneity of acute leukemias. These genetic events alter cellular pathways and functions, which may in turn affect the clinical phenotype of the disease and treatment response, thereby facilitating individualized targeted therapy.

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Diagnosis and Treatment of Childhood Acute Lymphoblastic Leukemia

17

Melinda Pauly and Lewis B. Silverman

Introduction

Acute lymphoblastic leukemia (ALL) is the most common malignancy of childhood, accounting for 25–30% of all pediatric cancer cases [1]. Leukemias arise from genetic changes that occur in a single lymphoid progenitor cell at various stages of maturation, resulting in a clonal expansion. The single-cell origin of ALL is demonstrated by the finding of clonal rearrangements of immunoglobulin (Ig) or T-cell receptor (TCR) genes in most lymphoblasts [2]. Malignant lymphoblasts possess the immunophenotypic and genetic characteristics of either B- or T-lymphoid precursors [3, 4]. The inability of these lymphoid progenitors to differentiate as well as their resistance to cell death leads to their accumulation in the marrow compartment and spread throughout the body. By the time of diagnosis, lymphoblasts have usually occupied much of the bone marrow microenvironment at the expense of normal hematopoietic cells, resulting in anemia, thrombocytopenia, and/or neutropenia. Effective treatment for the majority of patients consists of multiagent chemotherapy administered for 2–3 years, resulting in clonal eradication; the intensity of therapy is stratified based on presenting features, leukemia genetics, and early response to therapy.

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Incidence

Of the approximately 6600 new cases of ALL diagnosed in the USA per year, 57% of cases are under the age of 20 years and 15.6% of death from ALL occur in this age group [5]. ALL is the most common malignancy diagnosed in patients younger than 15 years, representing 23% of all cancers and 76% of leukemias in this age group [6]. Over the last 30 years, the incidence of ALL in the USA and Europe has steadily increased [1, 7]. The incidence of ALL is highest between ages 2 and 5 years, and is higher in boys than in girls (especially for T-ALL), although girls have a slightly higher (1.5 times) incidence in the first year of life [8].

Predisposing Factors

Risk factors for developing leukemia include family history, underlying genetic conditions, and (rarely) certain environmental exposures. For the vast majority of cases of childhood ALL, predisposing factors cannot be identified.

Fraternal twins and siblings of children with leukemia have a two- to fourfold greater risk of developing leukemia during the first decade of life [9, 10]. The concordance rate of ALL in monozygotic twins ranges from 5 to 25%, depending on subtype and age that the first twin develops leukemia. When one twin develops infant ALL (typically with a *KMT2A* gene rearrangement), ALL almost invariably develops in the other twin with a short latency (generally within a few months), likely reflecting transfer of ALL cells via the placental circulation [11–14]. The concordance rate in twins with ALL with the *ETV6-RUNX1* fusion is much lower at 10% and the postnatal latency period is longer, suggesting that cooperating genetic events are required postnatally for leukemic transformation in this subtype of ALL [15, 16].

Only a small proportion (<5%) of cases of ALL are associated with hereditary genetic abnormalities. The most common condition associated with the development of ALL is Down syndrome (trisomy 21). Children with Down syndrome

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have a 20-fold greater risk of ALL compared with non-Down syndrome children [17, 18]. The ALL observed in children with Down syndrome appears biologically distinctive, with a higher frequency of *CRLF2* overexpression and *JAK2* mutations, and a lower frequency of other recurrent genetic abnormalities, such as *ETV6-RUNX1*, high hyperdiploidy, *KMT2A*-rearrangement, and *BCR-ABL1* [19–23].

Other genetic disorders associated with an increased risk of ALL include ataxia telangiectasia and Bloom's syndrome [18, 24, 25]. Patients with ataxia telangiectasia have a 70-fold greater risk of leukemia, particularly T-ALL [26]. Nearly 90% of pediatric cases of low hypodiploid ALL (32–39 chromosomes), a rare subtype, have a TP53 mutation (compared with <5% of non-low-hypodiploid ALL); almost half of the TP53 mutations observed in low hypodiploid ALL are germline [27].

Genome-wide association studies (GWAS) have identified a number of germline polymorphisms associated with increased risk for developing childhood ALL. The first SNPs which GWAS technology identified as significantly associated with ALL were *ARIDB5, IKZF1,* and *CEBPE* [28, 29]. Additional variants in *CDKN2A, BMI1-PIP4K2A,* and *GATA3* have been identified and verified as increasing the host's risk for childhood ALL [30–32]. In addition, it is now known that specific susceptibility variants are associated with either molecular subtypes of ALL and/or ancestry. For instance, the risk alleles of *ARID5B*, a gene that encodes a transcriptional factor important in embryonic development, are associated with the development of the high hyperdiploid B-ALL subtype, and the susceptibility associated with *CEBPE* and *CDKN2A* polymorphisms is only seen in Europeans [33]. While a number of polymorphisms have been identified, the effect size of these genetic variants is relatively small, and very few childhood ALL cases are associated with them.

In utero exposure to diagnostic X-rays is associated with a slightly increased risk of ALL, proportional to the number of exposures [34]. The association between leukemia and maternal exposure to potential mutagens, parental use of medications and drugs, proximity to electromagnetic fields, parental smoking, exposure to petrochemical air pollution, and administration of vitamin K in the neonatal period have been studied, but findings have been contradictory and/or inconclusive [10, 35–40]. For instance, one study found that in utero exposure to DNA-damaging drugs, herbal medicines, or pesticides was significantly associated with infant leukemia with *KMT2A* rearrangements [41], but a Children's Oncology Group study found no association with pesticides and infant ALL [42].

Clinical and Laboratory Features at Diagnosis

Rarely ALL is detected during routine examination in the absence of signs or symptoms, but in most cases some symptoms are present for a few days to a few weeks prior to diagnosis. Patients typically present with the clinical signs of pancytopenia due to replacement of normal hematopoiesis by leukemic cells. Symptoms may include pallor, fatigue, or dizziness from anemia, and bruising and/or petechiae from thrombocytopenia. Fever is common and can be caused by infection or by pyrogenic cytokines released by the leukemic cells. In addition, leukemic infiltration of the marrow is often a painful process and patients may present with bone pain and arthralgias, frequently with a limp and complaints of back pain. Abnormalities of the bone, such as metaphyseal banding, periosteal reactions, osteolysis, osteosclerosis, or osteopenia, can be revealed by radiography in many patients. As these changes do not affect treatment and outcome, routine diagnostic imaging studies (except for chest radiograph to rule out mediastinal mass) are not necessary.

The complete blood count generally reveals abnormalities in more than one lineage; anemia, neutropenia, and thrombocytopenia are all usually present to various degrees. Hemoglobin is commonly <8 g/dL. Profound neutropenia (<500/mm^3) occurs in 40% of patients, putting them at a high risk of infection. Initial leukocyte counts may range from very low to extremely high; presenting leukocyte counts are >10,000/mm^3 in slightly over 50% of patients and >50,000/mm^3 in approximately 20% of patients. Patients with T-ALL are more likely to present with elevated leukocyte counts than those with B-ALL [43, 44]. Leukemic blast cells are frequently, but always, observed on peripheral blood smears, and their absence does not rule out a diagnosis of acute leukemia in a patient with pancytopenia. Hypereosinophilia, generally reactive, may be present at diagnosis; hypereosinophilia in B-ALL can also be associated with the t(5;14)(q31;q32) translocation involving the IL-3 gene on chromosome 5 and the Ig heavy-chain (IgH) gene on chromosome 14 [45].

Extramedullary sites of leukemic involvement include the liver, spleen, thymus, lymph nodes, and kidney. Hepatosplenomegaly is a common physical exam finding at diagnosis, as is lymphadenopathy. Painless enlargement of the scrotum can be a sign of testicular leukemia or hydrocele resulting from lymphatic obstruction; these two causes can usually be distinguished by ultrasound.

Leukemic infiltration of the thymus can lead to the development of an anterior mediastinal mass, observed on diagnostic chest X-ray in approximately 10% of newly diagnosed patients, almost always associated with T-cell immunophenotype. Anterior mediastinal masses can lead to life-threatening airway compression as well as superior vena cava (SVC) syndrome (facial and upper limb swelling, venous distension in neck, upper chest, and arms). Pleural and pericardial effusions can also be associated with mediastinal masses, which can exacerbate respiratory distress and cardiovascular compromise.

CNS involvement at diagnosis is usually asymptomatic and is diagnosed by cerebrospinal fluid (CSF) examination.

Lymphoblasts are identified morphologically at diagnosis in the CSF in over 20% of patients, most of whom have no neurologic symptoms [46]. Rarely, patients present with signs of increased intracranial pressure (vomiting, headache, papilledema, and lethargy) related to CSF pleocytosis or cranial nerve palsy related to leukemic infiltration. Ophthalmologic examination may reveal leukemic infiltration of the anterior ocular chamber, retinal hemorrhage and/or detachment, and optic nerve infiltration, especially in patients presenting with hyperleukocytosis.

Rapid turnover of leukemia cells at the time of diagnosis, especially in patients with high leukemic cell burden, can lead to elevations in serum uric acid, potassium, phosphorus, and lactate dehydrogenase (LDH). Patients with high levels of uric acid are at risk for the development of acute renal failure secondary to uric acid deposition in the kidney. Hypercalcemia at diagnosis has been observed but is uncommon; it appears to be related to elevated levels of parathyroid hormone-related peptide and has been associated with the rare t(17;19) translocation (*E2A-HLF*) [47]. Disseminated intravascular coagulation (DIC) may also occur at diagnosis, more commonly in T-ALL than B-ALL, and is only rarely associated with severe hemorrhage [48]. Coagulopathy has also been associated with the t(17;19) translocation [49].

Differential Diagnosis

Many of the presenting features of ALL are common to other childhood illnesses. Infection due to Epstein–Barr virus, cytomegalovirus, and other viruses can present with fever, lymphadenopathy, hepatomegaly, or splenomegaly, along with atypical lymphocytes that can be confused as lymphoblasts on peripheral blood smear. Patients with pertussis or parapertussis may have marked lymphocytosis, although composed by mature lymphocytes rather than lymphoblasts.

Patients with idiopathic thrombocytopenic purpura (ITP) can present with petechiae and bruising. In contrast to ALL, ITP typically presents with isolated thrombocytopenia, often with large platelets seen on blood smear, as opposed to abnormalities in other cell lines and smear findings suggestive of marrow infiltration observed with ALL.

Aplastic anemia may present with pancytopenia, but usually without the extramedullary findings frequently noted in ALL, such as hepatosplenomegaly and lymphadenopathy. Bone pain, arthralgia, and occasionally arthritis may mimic juvenile rheumatoid arthritis, rheumatic fever, other rheumatologic diseases, or osteomyelitis; because of the difficulty in distinguishing some rheumatologic conditions from ALL, bone marrow examination should be considered before initiating corticosteroid therapy. Solid tumors with extensive marrow metastatic involvement, including neuroblastoma and rhabdomyosarcoma, may also present similarly to ALL.

The definitive diagnosis of ALL requires morphologic examination of the bone marrow along with immunophenotyping. Cytogenetics and molecular analyses should be sent from diagnostic specimens for further classification of the leukemia. While the diagnosis of ALL can usually be made from a bone marrow aspirate, it is sometimes difficult to obtain an adequate aspirate sample (due to marrow fibrosis), in which case the diagnosis can be made by morphologic examination and immunohistochemical staining of a bone marrow biopsy specimen. For patients presenting with high peripheral blast count, peripheral blood flow cytometry may also be useful.

Diagnostic Classification

Morphology and Cytochemistry

ALL is diagnosed when more than 25% of the cells in the bone marrow are lymphoblasts. Morphologic analysis distinguishes three subtypes of ALL (L1, L2, and L3) as classified by the French–American–British (FAB) schema [50]. L1 lymphoblasts are small with scant cytoplasm. The cells are characterized by a large nucleus often with clefts along the membrane and prominent nucleoli. Approximately 90% of childhood ALL cases exhibit this morphology. In contrast, L2 lymphoblasts are much larger with more abundant cytoplasm and may resemble M1 myeloblasts. The L2 morphology is present in 5–10% of pediatric ALL. The distinction between L1 and L2 lymphoblasts, however, has no prognostic relevance with contemporary therapy [51]. L3 lymphoblasts occur in 1–2% of all cases; these cells exhibit vacuoles throughout deep blue cytoplasm. L3 lymphoblasts are nearly always associated with surface immunoglobulin expression and translocations involving the *MYC* gene, and are more effectively treated with regimens for advanced-stage Burkitt lymphoma.

Immunophenotype

The immunophenotype of ALL cells refers to the compilation of specific surface antigens which reveals both the lineage and maturation stage of the lymphoid progenitor cell from which the leukemia originated (Table 17.1). Lymphoblasts can be divided into two main immunophenotypic groups: B-ALL and T-ALL. Patients with B-ALL may be further subdivided into pro-B, early pre-B, pre-B, and mature B subtypes, as discussed below.

B-ALL

Approximately 80–85% of children with ALL present with B-ALL immunophenotype, characterized by the expression of the B-cell markers CD19, CD22, and CD79a [52]. The cells lack expression of cytoplasmic (or surface) CD3 and of myeloperoxidase (by cytochemistry and antibody staining). While

Table 17.1 Immunophenotypic subgroups of childhood ALL

Antigen expression (% of cases positive)										
Subtype	CD19	CD22	CD79a	CD10	CD7	CD5	CD3	cIgM	sIgM	sIg κ or λ	Prevalence (%)
Early pre-B	100	>95[a]	>95	95	5	0	0	0	0	0	60–65
Pre-B	100	100[a]	100	>95	0	<2	0	100[b]	0	0	20–25
Mature B	100	100[a]	100	50	0	0	0	>95	>95	>95	2–3
T	<5	0	30	45	100	95	100[a]	0	0	0	10-15

Source: Campana and Behm [52]
cIgM cytoplasmic immunoglobulin *μ* heavy chain; *sIgM* surface IgM; *sIg κ or λ* surface immunoglobulin *κ* or *λ* light chains
[a]Detectable only in the cytoplasm in some cases
[b]IgM heavy chains only

aberrant expression of myeloid antigens is not uncommon in B-ALL, co-expression of T-lineage antigens is rare [53].

Pro-B ALL is thought to be derived from a very immature B-cell precursor. It is most frequently observed in infants with ALL, especially those with *KMT2A* (*MLL*) rearrangements, and is characterized by absence of both CD10 surface expression and cytoplasmic immunoglobulin. In cases with *KMT2A* rearrangements, myeloid antigen co-expression is common [54, 55].

Early pre-B-ALL is the most common subtype of B-ALL seen in pediatric patients. Expression of CD10 and terminal deoxynucleotidyl transferase (TdT) is found in at least 90% of cases, and CD34 is expressed in more than 75% of cases [52]. CD20 (a marker of a more mature B-cell) can be detected at diagnosis in approximately 50% of cases [56], although its expression may increase during induction treatment [57]. Cytoplasmic and surface immunoglobulin are typically absent.

Pre-B-ALL, defined by the presence of cytoplasmic IgM heavy chains without detectable surface immunoglobulins, represents approximately 20–25% of cases of B-ALL [52]. In rare cases, IgM heavy chains without κ or λ light chains are also detectable on the cell surface [58], but can be distinguished from Burkitt-type leukemia by the absence of c-*MYC* translocations. Like early pre-B-ALL, pre-B-ALL cells usually express CD10 and TdT [52]. The *TCF3-PBX1* gene fusion is associated with pre-B immunophenotype; this cytogenetic abnormality is found in 20–25% of pre-B-ALL cases but in only 1% of cases with early pre-B-ALL [52, 53].

Mature B-cell ALL, which occurs in 1–2% of childhood ALL cases, is characterized by the expression of surface immunoglobulin. Cells are generally recognizable by FAB L3 morphology, as described above. CD20 is frequently expressed and CD34 is typically negative [52]. Mature B-cell ALL is characterized by the presence of c-*MYC* rearrangements caused by the translocation of this gene on chromosome 8 with one of the chromosomes containing an immunoglobulin gene, such as t(8;14)(q24;q32), t(2;8)(p12;q24), and t(8;22)(q24;q11) [59]. Mature B-cell ALL should be treated as advanced-stage Burkitt lymphoma rather than on regimens intended for childhood B-ALL [60].

T-ALL

Approximately 10–15% of children with ALL present with T-cell immunophenotype. Compared with B-ALL, T-ALL is more frequently associated with older age at diagnosis higher presenting leukocyte counts, and male predominance [44]. Mediastinal masses, when present in children with ALL, are almost exclusively observed in patients with T-cell immunophenotype.

The cell surface markers most consistently expressed in T-ALL are CD7 and CD3. Expression of the latter is confined to the cytoplasm in approximately two-thirds of cases [61]; approximately half of these cases also express cytoplasmic TCR proteins (TCRβ, TCRα, or both). In the remaining third of T-ALL cases, CD3 is expressed on the cell surface together with TCR proteins, TCRαβ, or, less commonly, TCRγδ [62]. Other markers usually expressed in T-ALL include CD2, CD5, and TdT; CD1a is detected in approximately half of the cases and two-thirds expressing CD4 and/or CD8 [52, 63]. Aberrant myeloid antigen expression may be seen in up to 15% of T-ALL [63].

Early T-cell precursor (ETP) ALL is a distinct subset of T-ALL that was first identified by gene expression profiling studies [64]. This subtype is characterized by a gene expression profile similar to that of normal thymic ETP cells, a population of recent immigrants from the bone marrow to the thymus which retains multi-lineage differentiation potential [65, 66]. ETP ALL expresses a specific immunophenotype that lacks CD1a and CD8 expression, has weak or absent CD5 expression, and expresses at least one stem cell or myeloid-associated antigen (e.g., CD34, CD117, CD13, CD33, and CD11b) [64]. ETP-ALL represents approximately 10–15% of childhood T-ALL cases and was initially thought to have an extremely poor prognosis [64, 67], although subsequent reports suggest that its outcome may not be significantly different than other cases of T-ALL [68].

Myeloid Antigen Co-expression

Up to 20–30% of lymphoblasts in childhood ALL co-express myeloid antigens. Myeloid antigen co-expression is associated with certain chromosomal abnormalities (both

favorable and unfavorable), such as *KMT2A* rearrangements, *BCR-ABL1*, and *ETV6-RUNX1*. Older studies suggested that myeloid co-expression was associated with an inferior outcome [69], but more recent reports have indicated that myeloid antigen co-expression lacks independent prognostic significance [70–72]. Distinction is made between ALL with myeloid co-expression and leukemia of ambiguous lineage or MPAL (mixed-phenotype acute leukemia), another leukemia subtype in which a predominant lineage cannot be determined. To be classified as MPAL, the 2008 WHO classification system requires that cells co-express at least two of the following three groups of lineage-defining antigens: [1] myeloid: myeloperoxidase or evidence of monocytic differentiation; [2] T-cell: cytoplasmic CD3; and [3] B-cell surface markers CD19, CD79a, CD22, and/or CD10 [73].

Table 17.3 Classification of ALL according to the 2008 revision of the World Health Organization classification of myeloid neoplasms and acute leukemia

B lymphoblastic leukemia
B lymphoblastic leukemia, not otherwise specified
B lymphoblastic leukemia with recurrent genetic abnormalities
B lymphoblastic leukemia with t(9;22)(q34;q11.2); *BCR-ABL1*
B lymphoblastic leukemia with t(v;11q23); *MLL* (*KMT2A*) rearranged
B lymphoblastic leukemia with t(12;21)(p13;q22); *TEL-AML1* (*ETV6-RUNX1*)
B lymphoblastic leukemia with hyperdiploidy
B lymphoblastic leukemia with hypodiploidy
B lymphoblastic leukemia with t(5;14)(q31;q32); *IL3-IGH*
B lymphoblastic leukemia with t(1;19)(q23;p13.3); *TCF3-PBX1*
T lymphoblastic leukemia

Source: Modified from Vardiman et al., 2009 [73]

Cytogenetics and Molecular Genetics

There are several recurrent cytogenetic abnormalities that have been identified in childhood ALL, many with important prognostic implications, as summarized in Table 17.2. The 2008 World Health Organization (WHO) classification of ALL is based on immunophenotype and cytogenetics, as shown in Table 17.3 [73].

Cytogenetic abnormalities in childhood ALL can be subdivided into those involving [1] abnormalities in chromosome number (ploidy) and [2] chromosomal translocations and gains/deletions of chromosomal segments.

Table 17.2 Recurrent cytogenetic abnormalities in childhood B-ALL and their relation to prognosis with contemporary therapy[a]

Abnormality	Frequency[a]	Prognostic impact
Hyperdiploidy 51–65 chromosomes	33%	Favorable
t(12;21)(p13;q22) [*ETV6-RUNX1*]	25%	Favorable
t(1;19)(q23;p13) [*TCF3-PBX1*]	4%	Neutral
t(9;22)(q34;q11)	3%	Unfavorable
iAMP21	2%	Unfavorable
KMT2A (11q23) rearrangements	2% [~80% of infants]	Unfavorable
Hypodiploidy (including near haplopidy, low hypodiploidy)	1–2%	Unfavorable
t(17;19)(q22;p13) [*TCF3-HLF*]	<1%	Unfavorable
IKZF1 deletions	15%[b]	Unfavorable

[a]Frequency from Moorman et al., 2010 [77], except frequencies of *IKZF1* deletions
[b]Frequency from Clappier et al. 2015 [110]

Abnormalities of Chromosomal Number

Hyperdiploid ALL

The most common recurrent chromosomal abnormality in childhood ALL is high hyperdiploidy (defined as 51–65 chromosomes, or DNA index $\geq$ 1.16), occurring in 25–30% of cases [74]. High hyperdiploidy is characterized by nonrandom gains of chromosomes, with trisomies and tetrasomies occurring most often (listed in order of frequency) in chromosomes 21, X, 14, 18, 4, 17, 10, and 8 [75]. High hyperdiploidy frequently occurs as the sole cytogenetic abnormality, without other karytotypic aberrations, but can also be observed along with chromosomal translocations, including *BCR-ABL1* and *TCF3-PBX1*. Whole-genome and whole-exome sequencing of high-hyperdiploid ALL revealed aberrations of receptor tyrosine kinase (RTK)/Ras pathway signaling (*KRAS*, *NRAS*, *FLT3*, *PTPN11*) and histone modifiers (including *CREBBP* and *WHSC1*) in the majority of cases [76]. High-hyperdiploid ALL occurs more frequently in children with "low-risk" presenting features (age 1–10 years, presenting leukocyte count <50,000/mm^3), and when it occurs in the absence of other prognostically significant abnormalities (such as *BCR-ABL1* fusion), it has been identified as an independent predictor of favorable outcome [75, 77]. High hyperdiploid patients with trisomies of chromosomes 4, 10, and 17 have been reported to have particularly favorable outcomes [75, 78].

Near triploidy (68–80 chromosomes) and near tetraploidy (>80 chromosomes) occur in approximately 1–2% of cases of childhood ALL, and are biologically distinctive from high hyperdiploidy [79, 80]. Compared with high hyperdiploidy, near trisomy/tetraploidy is more often associated with the *ETV6-RUNX1* fusion and more frequently occurs in T-ALL patients [79–81].

Hypodiploidy

Hypodiploidy (<45 chromosomes) is much less common than hyperdiploidy in childhood ALL, and has been associated with an unfavorable outcome [82, 83]. The chromosome loss is not random; disomies of chromosomes X/Y, 8, 10, 14, 18, and 21 are preserved in the majority of cases [84]. Rather than representing a single subtype of ALL, hypodiploid ALL consists of two distinct biologic subsets, near haploidy (24–31 chromosomes) and low hypodiploidy (32–39 chromosomes), which differ strikingly on a molecular basis from each other [27]. In near haploidy, genetic alterations in RTK/Ras pathway signaling (including *NRAS*, *KRAS*, *NF1*, *MAPK*, *FLT3*, and *PTPN11*) and the lymphoid transcription factor *IKZF3* are common, while low hypodiploidy is characterized by alterations in *TP53* (observed in 90% of cases), *IKZF2*, and *RB1*. Of note, almost half of the *TP53* mutations in pediatric low-hypodiploid ALL appear to be germline [27].

Doubling of the chromosome content in hypodiploid lymphoblasts can occur, resulting in a pseudo-hyperdiploid clone. These "masked hypodiploid" cases are biologically and prognostically indistinguishable from other cases of low hypodiploidy [27]. At diagnosis, both the hypodiploid and pseudo-hyperdiploid clone may be evident; however, the doubled hypodiploid clone may be the only one detectable at diagnosis, and can be confused with true hyperdiploidy. Given the very different expected outcomes of near-haploid/low-hypodiploid (poor prognosis) and high-hyperdiploid ALL (favorable prognosis), cases of masked hypodiploidy need to be distinguished from true high hyperdiploidy for risk stratification and treatment allocation. By karyotype, the "extra" chromosomes in masked hypodiploidy mostly occur as tetrasomies from the doubling of retained disomies, while in true high hyperdiploidy, trisomies are more common then tetrasomies [84].

Chromosomal Translocations and Gains/ Deletions of Chromosomal Segments

ETV6-RUNX1

The *ETV6-RUNX1* fusion is the most frequent chromosomal rearrangement in childhood ALL. It is found in approximately 20–25% of childhood ALL, occurring nearly exclusively in B-ALL [85]. It is caused by the t(12;21)(p13;q22) which juxtaposes the 5′ portion of the *ETV6* (*TEL*) gene and the nearly complete *RUNX1* (*AML1*) gene [86]. This translocation is usually cryptic by conventional karyotyping; the abnormality is typically detected only by fluorescence in situ hybridization (FISH) or reverse transcriptase-polymerase chain reaction (RT-PCR) [86–88]. The non-translocated *ETV6* allele is frequently deleted [81, 89]. Like high hyperdiploidy, *ETV6-RUNX1* is more frequent in children aged 1–10 years [87, 90, 91] and has been shown to be an independent predictor of favorable outcome [77, 90].

The widely expressed *ETV6* gene belongs to the *Ets* family of transcription factors and *RUNX1* encodes a transcription factor that binds DNA as a heterodimer with core-binding factor (CBF) β; both *ETV6* and *RUNX1* are essential for normal hematopoietic development [92, 93]. There is evidence that the gene fusion occurs prenatally, giving rise to a preleukemic clone that requires additional postnatal genetic events for the development of ALL. For instance, analyses of neonatal blood spots of healthy babies indicated that *ETV6-RUNX1* was detectable at a 100 times the frequency of this ALL subtype in the general population [94]. Additionally, studies of five monozygotic twin pairs with concordant *ETV6-RUNX1* ALL revealed that there was discordance within each twin pair for genome-wide copy number alterations considered "driver" mutations [95].

BCR-ABL1

BCR-ABL1-positive ALL (also known as Philadelphia chromosome-positive or Ph+ ALL) occurs in 3–5% of cases of childhood ALL. The *BCR-ABL1* fusion gene results from the t(9;22)(q34;q11), which juxtaposes the 5′ portion of *BCR* and the 3′ portion of *ABL1*, encoding a constitutively activated tyrosine kinase [96]. The fusion protein varies in size, depending on the breakpoint in the *BCR* gene. In ALL, the translocation occurs most often in the minor breakpoint cluster regions, resulting in a *BCR-ABL1* fusion protein of 190 kD (p190), while in chronic myelogenous leukemia (CML), the major breakpoint cluster region is typically involved, resulting in a larger fusion protein of 210 kD (p210) [97]. Deletions of *IKZF1*, a gene that encodes Ikaros, a lymphoid transcription factor, have been observed in approximately 70% of cases *BCR-ABL1*-positive ALL [98].

BCR-ABL1 positivity in childhood ALL is associated with older age at diagnosis and higher presenting leukocyte counts [99]. Historically, patients with this subtype had a significantly worse outcome than other children with ALL, and were typically allocated to hematopoietic stem cell transplant (HSCT) after achieving complete remission [77, 100]. More recent clinical trials have indicated that the addition of imatinib, a selective inhibitor of the *BCR-ABL1* tyrosine kinase, to an intensive chemotherapy backbone may improve the prognosis for pediatric *BCR-ABL1*-positive patients and obviate the need for HSCT in first complete remission for the majority of patients [101, 102].

BCR-ABL1-Like Subtype, IKZF1 Deletions, and CRLF2 Overexpression

Lymphoblasts from approximately 15% of pediatric ALL patients have been found to have a gene expression profile similar to *BCR-ABL1*-positive ALL, but lacking the *BCR-ABL1* fusion [103–107]. This subtype, termed *BCR-ABL1*-like

(or Ph-like) ALL, is more common in older children and adolescents, and has been associated with an adverse outcome [106, 107]. *BCR-ABL1*-like ALL is characterized by a high frequency of *IKZF1* deletions, as well as overexpression of cytokine receptor-like factor 2 (*CRLF2*). Detailed genomic analyses have identified kinase-activating alterations in over 90% of patients with *BCR-ABL1*-like ALL [106], suggesting the potential for targeted interventions with tyrosine kinase inhibitors. Kinase-activating alterations appear to primarily impact the ABL-class signaling pathway (with fusions involving *ABL1*, *ABL2*, *CSF1R,* or *PDGFRB*) and JAK-STAT signaling pathway (including rearrangements of *JAK2, EPOR,* and/or *CRLF2*) [106, 108, 109].

IKZF1 deletions, including deletions of the entire gene and deletions of specific exons, are present in approximately 15% of pediatric B-ALL, with increased frequency in older children and adolescents, those with higher presenting leukocyte counts, and patients with Down syndrome [104, 110, 111]. *IKZF1* deletions are detectable in the majority of patients with *BCR-ABL1*-positive ALL [98, 112], and in 40–60% of cases with the *BCR-ABL1*-like subtype [103, 106, 107]; however, many patients with *IKZF1* deletions have neither of these abnormalities [107]. Several studies have indicated that *IKZF1* deletions independently predict the outcome in B-ALL, even when controlling for other adverse features, including *BCR-ABL1* status and high levels of minimal residual disease (MRD) [107, 110, 113, 114]. *IKZF1* deletions have also been shown to predict inferior outcome in biologically favorable subtypes, such as high hyperdiploidy [110]. Some reports indicate that the presence of an *ERG* gene deletion, observed in 3–5% of cases of B-ALL, may abrogate the adverse prognostic significance of *IKZF1* deletions when the two co-occur [115, 116].

Genomic alterations in *CRLF2*, located on the pseudoautosomal regions of the sex chromosomes, have been identified in 5–10% of cases of B-ALL and in approximately 50% of cases of the *BCR-ABL1*-like subtype [117, 118]. They are strongly associated with *IKZF1* deletions and *JAK* mutations [118–121], and are more common in children with Down syndrome, occurring in 50–60% of cases [121, 122]. They have also been reported to be more common in Hispanic patients [119]. Two alterations have been described, both of which lead to *CRLF2* overexpression: (i) a cryptic chromosomal translocation involving the *CRLF2* gene and IgH locus on chromosome 14, and (ii) interstitial deletions juxtaposing *CRLF2* with the *P2RY8* promoter in pseudoautosomal regions of the sex chromosomes, resulting in a *P2RY8-CRLF2* fusion [117–120]. Although univariate analyses have suggested that *CRLF2* abnormalities may have adverse prognostic significance, these alterations have not been shown to be independent predictors of outcome in most studies when controlling for other factors, such as *IKZF1* deletion and *BCR-ABL1*-like status [107, 119].

KMT2A (MLL) Gene Rearrangements

Rearrangements involving the *KMT2A* (*MLL*) gene occur in approximately 5% of childhood ALL cases, but in up to 80% of infants with ALL. The most common rearrangement is the t(4;11)(q21;q23) which fuses the *KMT2A* (chromosome 11) and *AFF1* (chromosome 4) genes, but multiple other fusion partners have been identified. *KMT2A* translocations are also found in AML, but particular translocations show lineage predominance; for instance the t(4;11) is found most often in ALL while the t(9;11) is more common in AML.

In ALL cases, *KMT2A* rearrangements are associated with high presenting leukocyte counts, CD10-negative B-cell immunophenotype (pro-B), and myeloid antigen coexpression [123]. Even with intensified chemotherapy regimens, infants with *KMT2A* rearrangements have an adverse prognosis, particularly those who present at a very young age (<6 months) and with extremely high leukocyte counts [124]. The prognosis of older (non-infant) pediatric patients with *KMT2A* rearrangements is not as well established, but it appears that such patients fare better than infant *KMT2A*-rearranged patients but not as well as other non-infant B-ALL patients lacking these aberrations [77, 125]. There is also controversy regarding the prognostic significance of various fusion partners. For infants with *KMT2A* rearrangements, outcome appears similarly unfavorable regardless of fusion partner [124, 125], but for older children, some (but not all) studies have found that the t(4;11) confers a worse prognosis than other translocations [125]. In one report, the t(11;19), involving *KMT2A* and *MLLT1* (*ENL*) was associated with a poor outcome in infants (all with B-ALL) but appeared to be associated with a relatively favorable progress in non-infant patients with T-ALL [126].

Gene expression profile studies indicate that *KMT2A*-rearranged ALL is characterized by overexpression of a number of genes, including *FLT3, LMO2*, HOX genes (e.g., *HOXA9*, *HOXA5*, *HOXA4*, and *HOXC6*), *NRAS,* and *KRAS* [127, 128]. The unique biology of *KMT2A*-rearranged ALL appears to be driven primarily by epigenetic dysregulation rather than somatic mutations. Whole-genome sequencing has revealed that *KMT2A*-rearranged infant ALL has one of the lowest frequencies of somatic mutations of any cancer [127]; in non-infant *KMT2A*-rearranged ALL, frequent mutations in epigenetic regulators (but not other somatic mutations) have been identified [127]. Wild-type *KMT2A* possesses a methyltransferase domain which regulates expression of multiple genes, including HOX genes; *KMT2A* rearrangements lead to disruptions in *KMT2A*'s normal epigenetic function, resulting in overexpression of multiple genes through transcriptional dysregulation [129, 130].

TCF3 Rearrangements

Approximately 5% of pediatric B-ALL cases have the balanced t(1;19)(q23;p13) or the unbalanced der [19]t(1;19)

(q23;p13), both of which juxtapose the *TCF3* (*E2A*) gene on chromosome 19 and the *PBX1* gene on chromosome 1 [131–133]. The resulting *TCF3-PBX1* fusion protein contains the transcriptional activation domains of *TCF3* linked to the DNA-binding domain of *PBX1*, thereby inappropriately activating the transcription of genes normally regulated by *PBX1*, as well as reducing wild-type *TCF3* activity [134, 135].

The *TCF3-PBX1* fusion had previously been considered a predictor of inferior outcome [136], but with contemporary therapy, it lacks prognostic significance and in general is no longer considered a high-risk feature [131, 137]. Some investigators have reported a higher rate of CNS relapse in patients with the t(1;19) [138], but this has not been confirmed by others [131].

Another fusion observed in childhood ALL involving *TCF3* is the t(17;19)(q22;p13), resulting in the fusion of *TCF3* with *HLF*, a gene that encodes another transcription factor [139, 140]. The *TCF3-HLF* fusion is rare, occurring in fewer than 1% of pediatric ALL cases, and is associated with a very poor prognosis [47, 77, 141]. ALL with the *TCF3-HLF* fusion presents with disseminated intravascular coagulation and hypercalcemia [47, 141]. Genomic profiling has revealed that *TCF3-HLF*-positive ALL is characterized by deletions in genes involved in B-cell development (*PAX5*, *BTG1*, and *VPREB1*) and by mutations in RAS pathway genes (*NRAS*, *KRAS*, and *PTPN11*) [142].

Intrachromosomal Amplification of Chromosome 21 (iAMP21)

Intrachromosomal amplification of the RUNX1 gene on chromosome 21 (iAMP21) occurs in approximately 2% of childhood ALL (almost exclusively B-ALL), and has been associated with older age at diagnosis (median age approximately 10 years) and with lower presenting leukocyte count (less than 50,000 cells/mm^3) [77, 87, 143]. It is diagnosed primarily by FISH, and is defined as three or more extra copies of RUNX1 on a single chromosome 21 (a total of five or more RUNX1 signals per cell). Initial retrospective analyses suggested that children with iAMP21 had a markedly inferior event-free survival compared with other patients [144, 145]. Subsequent reports have indicated that, with more intensive treatment, patients with iAMP21 may not have as high a risk of relapse as suggested in these initial studies [143, 146, 147].

Genetic Subtypes of T-cell ALL

T-ALL can be subdivided into multiple genetically distinct subsets, but most do not appear to be prognostically significant and are not used for treatment stratification.

Translocations involving one of the T-cell receptor loci [chromosome 14q11 (TCRα and TCRδ), 7q34 (TCRβ), or 7p14 (TCRγ)] have been observed in approximately 35% of cases of T-ALL [148]. The translocations include rearrangements of transcriptional factors to the TCR loci that are often not visible by karyotype but can be identified by FISH. Some of the more common translocations involve rearrangements of *LMO1* (11p15), *LMO2* (11p13), *TAL1*(1p32), and *HOX11L2/TLX3* (5q35) to TCRδ, *TLX1* (10q24) to TCRδ or TCRβ, and *HOXA* (7p15) to TCRβ [149]. In addition, aberrant expression of these transcription factors can occur in the absence of T-cell receptor loci rearrangements; for instance, the *TLX3/HOX11L2* locus is recurrently translocated to T-cell regulatory sequences in the proximity of the *BCL11B* locus, small intrachromosomal deletions in chromosome 1p32 result in *TAL1* overexpression, and cryptic deletions in chromosome 11p13 can lead to activation of the *LMO2* oncogene [149].

Activating mutations in the *NOTCH1* gene are present in over 50% of T-ALL cases [150]. Additionally, *FBXW7* mutations, observed in about 15% of T-ALL, lead to constitutive activation of *NOTCH1* signaling by impairing degradation of activated *NOTCH1* [151]. The prognostic significance of *NOTCH1*-activating mutations is not clear. While some investigators have reported that these lesions are associated with a favorable early response (e.g., increased sensitivity to corticosteroid prophase) and/or a decreased risk of relapse, most studies have not demonstrated that these lesions have any prognostic significance [152–155].

Other common genetic lesions observed in T-ALL include deletions in the *CDKN2A* locus at chromosome 9p21, observed in 70% of cases, and deletions of *PTEN* (5–10% of cases), which is a negative regulator of the PI3K-AKT signaling pathway [156, 157]. Additional activating mutations of *PI3K* and *AKT* genes have also been reported, suggesting that up to 40–50% of T-ALL patients may have genetic alterations impacting this signaling pathway [156]. *ABL1* rearrangements occur in about 8% of T-ALL, including the *NUP214-ABL1* fusion (the most frequent *ABL1* abnormality), a cytogenetically cryptic, complex rearrangement seen on FISH on amplified episomes [158, 159].

The early T-cell precursor (ETP) subtype appears to be molecularly heterogeneous, but distinct from other cases of T-ALL. Compared with other T-ALL cases, ETP ALL has a lower rate of *NOTCH1* mutations and significantly higher frequencies of alterations in genes regulating cytokine receptors and RAS signaling, hematopoietic development, and histone modification [160].

Prognostic Factors

Several factors have been identified as significant predictors of outcome in childhood ALL, including age, presenting leukocyte count, immunophenotype, chromosomal abnormalities, presence of morphologically detectable lymphoblasts in

the spinal fluid at diagnosis, and early response to initial therapy [161]. These factors are used to stratify the intensity of therapy, with stronger, potentially more toxic treatments reserved for those presenting with adverse prognostic features. Ultimately, the prognostic significance of any factor is treatment dependent, and the importance of a particular presenting feature in predicting the outcome may vary, depending on the therapy delivered to that patient.

Age

Age at diagnosis is a long-established prognostic factor in B-ALL, but not T-ALL [162–165]. Its prognostic significance likely reflects age-related differences in the frequencies of various underlying biologic subsets. For instance, the two cytogenetic abnormalities associated with the most favorable outcome in childhood ALL, *ETV6-RUNX1* and high hyperdiploidy, occur most frequently in children aged 1–10 years, the age group with the best prognosis: approximately 80% of children with B-ALL diagnosed between the ages of 2 and 7 years have one of these two abnormalities [91].

Infants are the age group with the worst prognosis in pediatric ALL. Nearly 80% of infants present with rearrangements of the *KMT2A* gene, compared with <5% of older children [54, 55, 124, 162]. Even when treated with intensified regimens, infants with *KMT2A* rearrangements have an unfavorable prognosis, with long-term event-free survival rates less than 50% [124, 125, 166]. Infants whose leukemia lacks a *KMT2A* rearrangement fare better, with event-free survival rates that are closer to those observed in older children [124].

Older children and adolescents (10–21 years of age) with ALL also have a less favorable outcome than children aged 1–10 years at diagnosis, although not as poor as infants. Compared with younger children, adolescents with ALL more frequently present with higher risk biologic subtypes, including T-cell immunophenotype, *BCR-ABL1* fusion, *IKZF1* deletions, and *BCR-ABL*-like subtype, and less often with more favorable cytogenetic aberrations, such as high hyperdiploidy and the *ETV6/RUNX1* fusion [106, 107, 110, 162, 167]. Multiple retrospective studies have shown that adolescents fare better with pediatric ALL regimens than on treatments designed for adults with ALL [167–170].

Leukocyte Count

The initial peripheral blood leukocyte count is a significant predictor of treatment outcome, with outcomes worsening as the leukocyte count increases [161]. The relationship between leukocyte count and risk of subsequent relapse is more firmly established for B-ALL than T-ALL [43, 44, 171]. Based on a cutoff established by Cancer Therapy Evaluation Program (CTEP) of the National Cancer Institute (NCI), many clinical trials utilize a leukocyte count of 50,000 cells/mm^3 as the cutoff to differentiate between high versus low presenting leukocyte count [161].

Immunophenotype

Historically, immunophenotype was considered an important prognostic factor, with inferior outcomes observed in patients with T-ALL. However, if treated with more intensive regimens, children with T-ALL fare as well as those with B-ALL [44]. Myeloid antigen coexpression was previously thought to be associated with an inferior outcome, but more recent reports have indicated that it is not an independent prognostic factor [72, 172].

Central Nervous System (CNS) Disease at Diagnosis

Approximately 15–20% of children with ALL present with detectable lymphoblasts in their cerebrospinal fluid [173, 174]. Some children, such as those diagnosed in infancy and those with T-cell ALL, have a higher incidence of morphologically evident CNS leukemia at diagnosis [162].

CNS status at presentation is usually classified as CNS-1 (no blast cells in spinal fluid), CNS-2 (fewer than five leukocytes per microliter with blast cells), and CNS-3 (five or more leukocytes per microliter with blast cells or cranial nerve palsy) [161]. With more frequent dosing of intrathecal chemotherapy, the prognosis of patients with CNS-2 status appears similar to those who are CNS-1 [174, 175]. CNS-3 status at diagnosis (observed in approximately 5% of patients) is associated with a higher risk of relapse (both CNS-involved and marrow-only), and is typically treated with more intensive systemic and CNS-directed therapies [174].

Traumatic lumbar punctures with lymphoblasts on cytospin have also been associated with an adverse prognosis [174, 176]. Like those with CNS-2 status, patients with traumatic lumbar punctures with lymphoblasts at diagnosis may also benefit from additional doses of intrathecal chemotherapy early in treatment [174].

Chromosomal Abnormalities and Other Genetic Lesions

Recurrent chromosomal abnormalities in childhood ALL are detailed above. Several of these have been shown to be significant predictors of outcome. Two abnormalities, the cryptic t(12;21) (*ETV6/RUNX1* fusion) and high hyperdiploidy (51–65 chromosomes or a DNA index ≥1.16), have each been associated with a favorable prognosis [77].

The most favorable outcomes in high-hyperdiploid ALL patients have been associated with the presence of trisomies of chromosomes 4, 10, and 17 [177–179]. Both *ETV6-RUNX1* and high hyperdiploidy occur more most commonly in younger, non-infant patients with B-ALL, with decreased frequency in adolescents, and are usually mutually exclusive [81, 91].

Chromosomal abnormalities associated with an unfavorable prognosis include low hypodiploidy [82, 83, 180], rearrangements of the *KMT2A* (*MLL*) gene [125, 180, 181], and *BCR-ABL1* fusion (Philadelphia chromosome) [77, 100]. The use of imatinib and other tyrosine kinase inhibitors, given in conjunction with intensified chemotherapy, appears to have favorably impacted the outcome of *BCR-ABL1*-positive ALL [101, 102]. Intrachromosomal amplification of chromosome 21 (iAMP21) has also been associated with a higher risk of relapse [144, 145], although the adverse prognostic significance of this abnormality appears to be abrogated when patients are treated on more intensive, "high-risk" therapy [143, 146, 147].

Patients with *BCR-ABL1*-like ALL (defined by gene expression profile) and/or *IKZF1* gene deletions (each representing approximately 15% of B-ALL, with a large overlap between the two groups) have an inferior outcome; each has been shown to be an independent adverse prognostic factor [106, 107, 110, 113, 114].

Early Response to Therapy

The rapidity with which a patient responds to initial chemotherapy is a significant predictor of long-term outcome. Early response to therapy has been evaluated using morphologic measures (residual microscopically detectable disease in blood or marrow) and more sensitive minimal residual disease (MRD) techniques to quantitate submicroscopic levels of disease, such as flow cytometry, PCR, and next-generation sequencing (NGS).

Morphologic Response to Therapy

On trials run by the Berlin-Frankfurt-Munster (BFM) group and several other clinical trial consortia, patients begin treatment with 1 week of corticosteroid monotherapy (and one dose of intrathecal methotrexate) prior to beginning multiagent induction chemotherapy; poor peripheral blood response at the end of that week (defined as an absolute blast count of 1000/mm^3) is an independent predictor of adverse outcome [182]. Similarly, the persistence of leukemia in bone marrow specimens obtained 7 or 14 days after beginning multiagent chemotherapy strongly correlates with increased relapse risk [183], although intensification of therapy can abrogate the adverse prognostic significance of slow early morphologic marrow response [184].

Patients who require two or more cycles of induction chemotherapy to achieve complete remission (CR) have a much worse prognosis than those who achieve CR after the first induction attempt [185–187].

Minimal Residual Disease

Minimal residual disease (MRD) evaluation involves the measurement of very low levels of leukemia using sensitive assays, such as specialized multiparameter flow cytometry, PCR, or NGS techniques. Leukemic cells are identified using targets identified at diagnosis, including leukemia-specific immunophenotypes (for flow cytometry-based assays), chromosomal translocations, or lymphoblast-specific immunoglobulin or T-cell antigen receptor gene rearrangements (for PCR- and NGS-based assays). Using these techniques, leukemia cells have been identified at levels as low as 1 in 1000 to 1 in 100,000 cells [188–192].

Many studies have demonstrated that MRD status at early time points in treatment is a significant and independent predictor of long-term outcome for patients with both B-ALL and T-ALL [188, 193–197]. For patients achieving a morphologic remission at the end of the first month of treatment, those with higher levels of marrow MRD at that time point have a higher risk of relapse than those with lower or undetectable MRD [188, 193–195, 197–201]. MRD levels measured in the peripheral blood as early as 8 days after starting multiagent chemotherapy have also been shown to be prognostically significant, especially in patients with B-ALL presenting with standard-risk features (age between 1 and 10 years, leukocyte count <50,000/mm^3) [199].

MRD levels obtained 10–12 weeks after the start of therapy (at the end of the second phase of treatment) have also been shown to be prognostically important; patients with high levels of MRD at this time point have a significantly inferior EFS compared with other patients [200–202]. The AIEOP-BFM group has defined three prognostically distinct groups of patients based on MRD measurements: (1) patients with low end-induction MRD (best outcome); (2) patients with high end-induction MRD but low MRD after the second phase of treatment (intermediate outcome); and (3) patients with persistently high MRD after the second phase of treatment (worst outcome) [200, 201].

Intensifying therapy for patients with high MRD has been shown to improve the outcome [203, 204]. MRD measurements, in conjunction with other presenting features, have also been used to identify subsets of patients with an extremely low risk of relapse. The Children's Oncology Group reported a very favorable outcome (5-year EFS of 97%) for B-ALL patients with non-high-risk presenting features (age between 1 and 10 years, leukocyte count <50,000/mm^3, CNS-1 status, and either high hyperdiploidy with favorable trisomies or *ETV6-RUNX1* fusion) and MRD levels

of less than 0.01% at both day 8 (from peripheral blood) and end induction (from bone marrow) [199].

Other Prognostic Factors

Gender

In some studies, boys appear to fare slightly worse than girls. This observation cannot be entirely explained by the rates of isolated testicular relapse, which are relatively low with contemporary treatment regimens [205, 206].

Race and Ethnicity

Lower event-free survival (EFS) rates have been reported for African-American, Hispanic, and Native American patients, even after adjustment for differences in prognostically significant presenting features [207–209]. This finding may be in part related to differences in the frequency of prognostically relevant biologic subtypes amongst racial and ethnic groups. For example, compared with Caucasians, African-American children have a higher incidence of T-ALL and lower rates of high hyperdiploidy [210]. Hispanic children also have a lower frequency of *ETV6-RUNX1*-rearranged ALL [211], and, in one study, were noted to have a higher incidence of ALL with *CRLF2* rearrangements, a finding that is associated with the *BCR-ABL1*-like subtype [119]. Lower rates of adherence to oral 6-mercaptopurine, a critical component of ALL treatment, have also been observed in African-American, Hispanic, and Asian-American patients [212, 213].

Down Syndrome

Results from several studies have indicated that children with Down syndrome have inferior outcomes compared with other pediatric ALL patients. Down syndrome patients have been reported to have both higher rates of treatment-related mortality (with significantly increased risk of infections and other treatment complications) and relapse [23, 214–216]. The increased risk of relapse may be due in part to the lower frequency of favorable biologic features (such as *ETV6-RUNX1* and high hyperdiploidy) observed in Down syndrome patients [23, 214, 216]. ALL arising in Down syndrome patients is characterized by a higher incidence of *IKZF1* deletions, *CRLF2* aberrations, and *JAK* mutations [19, 111, 122]; the presence of *IKZF1* deletions (but not *CRLF2* aberrations and *JAK* mutations) has been associated with an inferior outcome in Down syndrome patients [111, 217].

Treatment Adherence

Poor adherence to oral 6-mercaptopurine (6-MP), a key component of maintenance therapy in childhood ALL, is an important predictor of relapse [212, 213, 218]. Using an electronic cap to record the date and time of 6-MP bottle openings, investigators from the Children's Oncology Group (COG) demonstrated that there was a progressive increase in relapse rate with decreasing adherence that remained statistically significant after adjusting for NCI risk classification, chromosomal abnormalities, and other prognostically relevant variables. When 6MP adherence was lower than 90%, the risk of relapse was nearly fourfold increased [212]. Factors associated with higher risk of nonadherence included older age (≥12 years), non-white race/ethnicity, low annual household income, low parental education, single-parent households, and absence of a routine surrounding pill taking [212, 213].

Treatment

Historical Background

Over the last several decades, there has been a dramatic improvement in the prognosis of children with ALL. Improvement in cure rates can be attributed to many factors, including [1] identification of active agents and development of complex chemotherapeutic regimens designed to achieve clonal eradication and prevent emergence of drug-resistant clones; [2] improvements in supportive care; [3] recognition of the central nervous system (CNS) as a sanctuary site; and [4] identification of prognostic factors and application of risk-adapted therapy.

Prior to 1947, when the first complete remission in childhood ALL was attained by Farber and colleagues using the folate antagonist aminopterin [219], the disease was uniformly fatal with a median duration of survival of 2 months from the time of diagnosis [220]. During the 1950s, drugs such as 6-mercaptopurine, methotrexate, and corticosteroids were found to be active and induced complete remissions in the majority of patients, but cure rates remained very low due to extremely high rates of relapse [221–224]. Additional active drugs were introduced in the 1960s and 1970s, including the anthracyclines (doxorubicin and daunorubicin), L-asparaginase, and epipodophyllotoxins (etoposide and teniposide) [225–227].

In the 1960s, as effective systemic chemotherapy combinations were identified, the incidence of the CNS as an initial site of relapse became increasingly more common [228, 229]. It was hypothesized that leukemia cells, even if not morphologically evident, were present in the CNS in all patients, and that these cells were protected by the blood-brain barrier from many of the systemically administered chemotherapy agents used at the time. Thus, the concept of the CNS as a sanctuary site emerged, prompting the inclusion of CNS-directed therapy to prevent relapse. With the introduction of cranial radiation to treat subclinical CNS

leukemia in the 1970s, long-term disease-free survival rates in childhood ALL dramatically increased to 50% [230]. Although nearly all pediatric patients are now treated without prophylactic cranial radiation, the inclusion of CNS-directed therapies (such as intrathecal chemotherapy and high-dose methotrexate) remains a universal component of all successful treatment regimens.

Risk-Adapted Therapy

After the addition of CNS-directed therapy improved cure rates in the 1970s, investigators compared presenting features in patients who relapsed and those who had not to establish clinically relevant prognostic factors. Subsequent clinical trials used these prognostic factors to stratify therapy. More intensive therapy was administered to those patients considered to be at the highest risk of relapse. In contrast, some of the more morbid components of therapy were modified or eliminated for those children considered to have the best prognosis. The goal of risk-adapted therapy is to treat away adverse presenting features, so that higher and lower risk patients have similar cure rates.

For many years, pediatric clinical trials consortia applied prognostic factors differently when defining risk categories. A more uniform approach to risk classification was proposed and agreed upon at an NCI-sponsored workshop held in 1993 [161]. For patients with B-ALL, the NCI standard-risk category was defined as age between 1 and 10 years and initial leukocyte count lower than 50,000/mm^3. The remaining patients were considered to have NCI high-risk ALL. Other characteristics used by cooperative groups to classify patients as high risk include T-cell phenotype, CNS-3 status at diagnosis, and high peripheral blast count after a week of steroid monotherapy (and a single dose of intrathecal methotrexate), cytogenetics, and MRD levels obtained after the first one or two treatment phases into risk group stratification. In some cases, only cytogenetics and early response (as assessed by MRD), and not other factors, such as age and leukocyte count, are considered when assigning risk groups [200].

Treatment Phases

In general, treatment regimens for children with ALL consist of 2–3 years of multiagent chemotherapy. Treatment consists of the three main parts: (1) remission induction, (2) post-induction consolidation, and (3) continuation (or maintenance). CNS-directed therapies are included throughout all phases. With contemporary regimens, event-free survival rates exceed 80%, and overall survival rates are approximately 90% [200, 204, 231, 232] (Table 17.4).

Table 17.4 Results of selected clinical trials in childhood ALL

Study	Time period	No. of patients	5-year EFS (%)	5-year OS (%)	References
AIEOP-BFM 2000[a]	2000–2006	4016	80[b]	92[b]	[200]
COG	2000–2005	7153	–	90	[232]
DCOG ALL-10	2004–2012	778	87	92	[204]
DFCI 05-001	2005–2010	551	85	91	[231]
NOPHO ALL-2000	2002–2007	1023	79	89	[372]
SJCRH Total XV	2000–2007	498	86	93	[138]
MRC UK-ALL 2003	2003–2011	3126	87	92	[203]

EFS Event-free survival, *OS* Overall survival, *AIEOP-BFM*, Associazione Italiana di Ematologia Pediatrica-Berlin Frankfurt-Munster; *COG* Children's Oncology Group, *DCOG* Dutch Childhood Oncology Group, *DFCI* Dana–Farber Cancer Institute ALL Consortium, *NOPHO* Nordic Society of Pediatric Hematology and Oncology, *SJCRH* St. Jude Children's Research Hospital, *MRC UK* Medical Research Council United Kingdom
[a]B-ALL only
[b]7-year estimates
[c]5-year overall survival

Remission Induction

The goal of the first phase of treatment, remission induction, is to induce complete morphologic remission. Complete remission is defined as attainment of a bone marrow with normal cellular elements but fewer than 5% lymphoblasts, return of normal peripheral blood counts, and resolution of other bulk sites of disease. The remission induction phase typically lasts for 4–5 weeks, and consists of glucocorticoid (prednisone, prednisolone, or dexamethasone), vincristine, and L-asparaginase; some regimens include an anthracycline (daunorubicin or doxorubicin) for all patients while others reserve its use for higher risk patients. About 1–2% of patients die of disease- or treatment-related complications during the first month of treatment, and about 1–2% fail to fully respond and have morphologically detectable disease at the completion of the remission induction phase [231, 233, 234]. Induction failure rates tend to be higher in patients with high presenting leukocyte counts and/or T-cell phenotype [185, 186]. Overall, more than 95% of pediatric patients with newly diagnosed ALL achieve complete remission at the end of the first month of treatment, with slightly lower rates in T-ALL compared with B-ALL [138, 164, 165, 231, 235–244].

Several randomized trials have compared two glucocorticoids, prednisone and dexamethasone, during remission induction and subsequent treatment phases. Potential advantages of dexamethasone include more potent in vitro

antileukemic activity, higher free plasma levels, and better CNS penetration [245–247]. In nearly all of the randomized trials, dexamethasone was associated with superior event-free survival [234, 248, 249], although in one trial (which closed early due to toxicity concerns with dexamethasone), no advantage was demonstrated for adolescents [250]. Dexamethasone during the remission induction phase has also associated with higher rates of infection, myopathy, hyperglycemia, and behavioral issues [234, 248–250].

Most patients with initial induction failure will eventually achieve complete remission; however the chance for subsequent relapse is quite high, leading to low rates of long-term survival [185–187]. Allogeneic hematopoietic stem cell transplantation (HSCT) after complete remission is achieved (as opposed to continued chemotherapy) may improve the outcome of patients with initial induction failure [251]. In a large retrospective series, a trend for superior overall survival with allogeneic HSCT compared with chemotherapy alone was observed in patients with initial induction failure and either T-ALL (any age) or B-ALL and age greater than 6 years [187].

Post-induction Consolidation

After achieving complete remission, patients typically receive several phases of treatment designed to further decrease levels of residual disease. The intensity of post-induction consolidation is stratified by risk group, with higher risk patients receiving stronger therapy.

A commonly used post-induction consolidation regimen was first introduced by the German Berlin-Frankfurt-Munster (BFM) study group [182], and this scheme has subsequently been adopted by several other large cooperative groups, with variations in some of the doses and agents used. The BFM-type consolidation regimen generally includes (1) a "consolidation" course consisting of cyclophosphamide, low-dose cytarabine, and a thiopurine (mercaptopurine or thioguanine), followed by (2) multiple doses of either high-dose or escalating doses of methotrexate with or without leucovorin rescue, and then (3) a reinduction (or delayed intensification) course, which typically include the same agents used during the initial induction/consolidation cycles [182]. This backbone has been modified on some protocols to eliminate or truncate some of the chemotherapy courses for lower risk patients, and intensified for high-risk patients by more doses of some agents (such as vincristine, pegaspargase, and methotrexate) and/or a second delayed intensification phase [184, 242, 252–256]. Alternative post-induction regimens have been adopted by some groups, with similarly favorable outcome results as those achieved using the BFM-type backbone. For instance, the consolidation phase on trials conducted by the DFCI ALL Consortium includes 20–30 weeks of consecutively dosed L-asparaginase along with frequent pulses of vincristine and corticosteroid, and doxorubicin for higher risk patients [231, 257, 258].

Continuation (Maintenance)

A standard feature of all treatment regimens for childhood ALL is a prolonged continuation or maintenance phase, consisting of daily mercaptopurine and weekly low-dose methotrexate. The importance of this phase is highlighted by results of studies indicating that patients who are compliant with less than 95% of their prescribed mercaptopurine doses have a significantly higher risk of subsequent relapse [212, 213, 218]. Pulses of vincristine and corticosteroid are frequently added to this maintenance backbone, although their benefit remains controversial [259–262]. When vincristine/corticosteroid pulses are used, it appears that dexamethasone is superior to prednisone [248, 249, 258], but also associated with increased risk of behavioral problems and skeletal toxicities [249, 258].

6-Thioguanine has been investigated as an alternative to 6-mercaptopurine during the continuation phase, with conflicting results regarding its impact on event-free survival [263–265]. The use of 6-thioguanine during maintenance has been associated with significant hepatotoxicity, including veno-occlusive disease and cirrhosis, as well as higher remission death rates, primarily caused by infection [263, 264], and so it is not typically used in this phase.

Approximately 0.5–1% of patients have an inherited homozygous deficiency of thiopurine *S*-methyltransferase (TPMT), which catalyzes the inactivation of mercaptopurine [266]. These patients are at increased risk for acute hematologic and hepatic toxicities when given standard doses of mercaptopurine, and can only tolerate much lower dosages [267, 268]. Patients who are heterozygous for this mutation (approximately 10% of the population) have intermediate levels of enzyme activity, generally tolerate mercaptopurine better than those with homozygous deficiencies, but still require dose reductions more frequently than patients who do not carry any mutant allele [267, 268]. Polymorphisms of the *NUTD15* gene, observed most frequently in Hispanic and East Asian patients, have also been associated with extreme sensitivity to mercaptopurine, necessitating significant dose reductions to avoid severe hematologic and hepatic toxicity [269].

On most treatment protocols, maintenance chemotherapy is administered until patients have received a total of 2–2.5 years of treatment from the time of diagnosis. Previous studies suggested that boys might benefit from a more prolonged continuation phase [270], so on some regimens, boys are treated for an additional year; however, the benefit of this approach with more contemporary regimens is not clear. Attempts to shorten therapy duration from 2 years have not been successful. In a randomized comparison of 18 versus 24 months of treatment, patients receiving the shorter duration had a higher rate of relapse [271]. Similarly, very high relapse rates were observed in a nonrandomized trial on which patients received intensified therapy for only

12 months, suggesting that truncated therapy, even if intensive, is inadequate for most children with ALL [272].

CNS-Directed Therapy

Because the central nervous system (CNS) is a sanctuary site into which many of the systemically administered agents used to treat childhood ALL do not effectively penetrate, treatment that is specifically directed at treating CNS leukemia is an essential component of all treatment regimens. Options for CNS-directed therapies include cranial radiation, intrathecal chemotherapy, and CNS penetrant systemic chemotherapy, such as high-dose methotrexate with leucovorin rescue, escalating-dose methotrexate, and dexamethasone. The type of CNS-directed therapy that is used is based on a patient's risk of CNS relapse, with higher risk patients receiving more intensive treatments.

Radiation therapy was the first treatment approach successfully used to prevent CNS relapses. In the 1960s and 1970s, studies performed at St. Jude Children's Research Hospital (SJCRH) documented the effectiveness of CNS radiation (cranial or craniospinal) in children with ALL [273, 274]. Subsequent studies demonstrated that 2400 cGy cranial radiation with intrathecal methotrexate was as effective in preventing CNS relapse as craniospinal radiation without intrathecal chemotherapy [275, 276]. Because craniospinal radiation was associated with increased toxicity, including excessive myelosuppression and spinal growth retardation, cranial radiation administered with intrathecal chemotherapy became the standard form of CNS treatment in the 1970s. Increased recognition of late effects associated with 2400 cGy cranial radiation led to the use of a lower dose (1200–1800 cGy) in trials conducted in the 1980s and 1990s [258, 277, 278].

The proportion of patients receiving cranial radiation has decreased significantly over the last few decades. With contemporary regimens, nearly all pediatric patients are treated without cranial radiation, relying on other CNS-directed therapies, including multiple doses of intrathecal chemotherapy, for CNS prophylaxis. When it is used, radiation is administered only to those patients considered to be at highest risk of subsequent CNS relapse, such as those with CNS-3 status at diagnosis and T-ALL patients with high presenting leukocyte counts and/or slow early response to initial therapy.

Several nonrandomized studies have been conducted in which cranial radiation was omitted for all patients, regardless of risk group status [138, 279, 280]. These trials intensified other CNS-directed therapies for higher risk patients, including additional doses of high-dose methotrexate and/or high-dose cytarabine and increased frequency of intrathecal chemotherapy. Predictors of subsequent CNS relapse on these trials included T-cell phenotype and the presence of blasts in spinal fluid at diagnosis [138]. In a meta-analysis of aggregated data from more than 16,000 patients treated between 1996 and 2007 by ten cooperative groups, only patients with CNS-3 status at diagnosis appeared to benefit from cranial radiation therapy [281]. In this analysis, CNS-3 patients who received cranial radiation had a significantly lower rate of relapses involving the CNS compared with nonirradiated CNS-3 patients; however, the overall 5-year mortality rate for CNS-3 patients was similar whether or not they received radiation.

Treatment Sequelae

As cure rates for childhood ALL have improved over the last several decades, late effects related to the disease and its treatment have become increasingly evident. A number of late effects have been documented in long-term survivors, including neurocognitive sequelae, short stature, obesity, cardiac dysfunction, cataracts, osteonecrosis, and second malignant neoplasms.

Neurocognitive Late Effects

Long-term neurocognitive sequelae have been well documented in survivors of childhood ALL. The frequency and severity of impairments vary by treatment and patient characteristics. Children treated at a younger age, and, in some studies, female patients, are at higher risk for developing neurocognitive late effects [282–288]. The most severely impaired long-term survivors are those who received relatively high cranial radiation doses (24–28 Gy) in the 1970s. Low and low average intelligence quotients (IQs) have been frequent findings in these patients [282, 283, 289], and they also exhibit a high frequency of neuropsychological deficits, including a slow speed of processing information, distractibility, and difficulty in dealing with complex or conceptually demanding material [285, 290]. Long-term survivors treated with lower dose (18 Gy) radiation, especially those who were 3 years or older at diagnosis, appear to fare better, with less severe neurocognitive late effects. In some studies, this group of survivors does not demonstrate any significant cognitive deficits, although subtle effects can be observed in some of these patients with detailed neuropsychological testing [284, 291].

Intrathecal chemotherapy and CNS-penetrant systemic treatments (such as high-dose methotrexate) also appear to contribute to neurocognitive late effects. There is evidence that cognitive deficits are present in long-term survivors treated without cranial radiation [291–294], but these deficits do not tend to be severe and their neurocognitive function is generally within the normal range [288, 295]. In most studies of long-term survivors, nonirradiated patients have

fewer and less severe impairments than those who received cranial radiation [283, 296], although with current chemotherapy regimens and lower doses of radiation, differences between the two groups may be subtle [291]. For nonirradiated patients, greater treatment intensity of systemic and intrathecal chemotherapy has been associated with a higher frequency of neuropsychologic deficits and difficulties at school [297].

Skeletal Toxicities

Osteonecrosis, which is observed in 5–10% of children treated for ALL, is a disabling bone toxicity resulting from treatment with glucocorticoids. It is typically diagnosed during the maintenance phase or soon after the completion of therapy, frequently involves multiple joints, and can lead to chronic pain and loss of function, sometimes requiring joint replacement and other surgeries [298–300]. Rates of osteonecrosis are significantly higher in adolescents than in younger children [258, 298, 300]. Adults with ALL do not seem to have as high an incidence of symptomatic osteonecrosis as teenagers, suggesting that the hormonal and physiologic changes of puberty may render adolescents more susceptible to this complication [301]. In addition to age, other risk factors for the development of osteonecrosis include higher total doses of glucocorticoids, female sex, and high body mass index [298, 300]. In some studies, dexamethasone (when used instead of prednisone) has been associated with a higher risk of osteonecrosis, particularly in adolescents [299].

Several studies have shown that children with ALL develop osteopenia during therapy [302, 303], most likely secondary to glucocorticoid exposure, resulting in an increased risk for fractures during and immediately after treatment [304, 305]. It appears that bone mineral density improves once therapy is completed, although some degree of residual osteopenia may persist [306–309].

Cardiac Late Effects

Anthracyclines, such as doxorubicin and daunorubicin, have been associated with cardiotoxicity in long-term survivors, including left ventricular wall thinning and depressed contractility [310]. Patients treated at a young age, females, and those with Down syndrome appear to be more vulnerable to developing anthracycline-associated cardiac toxicity [311, 312]. The severity of cardiac dysfunction is correlated with higher cumulative doses of anthracycline and higher dose rates [311–314]. Over the last few decades, therapeutic regimens have been modified so that patients receive lower cumulative dosages of anthracyclines; as a consequence, symptomatic congestive heart failure has become increasingly uncommon and now only rarely occurs in long-term survivors of childhood ALL [312, 314]. However, asymptomatic echocardiographic abnormalities can still occur, sometimes developing many years after completion of therapy, and may be progressive over time [315]. Randomized clinical trials have demonstrated that use of the cardioprotectant agent dexrazoxane can reduce the frequency of long-term cardiotoxicity in patients receiving relatively high doses of anthracycline without adversely impacting event-free survival rates [316–318].

Second Malignant Neoplasms

Long-term survivors of childhood ALL are at risk for developing second malignant neoplasms (SMNs), including brain tumors, acute myelogenous leukemia (AML), non-Hodgkin's lymphomas, and carcinomas of the parotid and thyroid glands [319–321]. The overall cumulative incidence of SMNs reported in the literature ranges from 1 to 6%, depending on the treatment regimen and length of follow-up [319, 320]. In a retrospective study of 2169 patients treated between 1962 and 1998 (median follow-up 18.7 years), the overall cumulative incidence of SMN was approximately 4% at 15 years and 11% at 30 years [320]. When benign neoplasms, such as basal cell carcinoma and meningioma, were excluded from that analysis, the cumulative incidence of SMNs at 30 years was approximately 6%.

Malignant gliomas and nonmalignant meningiomas occur almost exclusively in patients who received cranial or craniospinal radiation [319, 320]. The cumulative incidence of malignant glioma in irradiated survivors appears to plateau approximately 15–20 years after diagnosis; conversely, even with 30 years of follow-up, a plateau in the incidence of meningiomas has not been observed [320]. Cranial radiation has also been associated with the development of vascular malformations, which can lead to neurological symptoms and intracranial hemorrhage [322]. Rates of secondary AML are increased in patients who receive higher total (and/or more frequent) doses of epipodophyllotoxins and alkylating agents [323–325]. Some studies have suggested that secondary leukemia risk may also be increased in patients with homozygous or heterozygous deficiencies in thiopurine methyltransferase (an enzyme involved in the metabolism of mercaptopurine) [326].

Relapse

Approximately 15% of children with ALL who achieve complete remission will subsequently relapse. Relapses tend to occur during the first 5 years after initial diagnosis, but can

occur as late as 10 years [327]. The most common site of relapse is the bone marrow, with or without overt extramedullary involvement; more uncommonly, relapse can sometimes only be detectable only in an extramedullary site, such as the CNS or testes.

Duration of initial remission is one of the most important prognostic factors at the time of relapse, with significantly worse outcomes observed in patients with shorter initial remissions [328–330]. Site of relapse also has prognostic importance, with superior outcomes observed for patients experiencing isolated extramedullary relapses compared with marrow relapses [329, 330]. Some studies have suggested that patients with combined marrow and extramedullary relapses have a better prognosis than those with isolated marrow relapses [328, 330], although this has not been consistently demonstrated [329]. T-cell phenotype and age older than 10 years at initial diagnosis have also been associated with an adverse prognosis after relapse [328–330], as has response to initial reinduction chemotherapy assessed by sensitive minimal residual disease (MRD) tests [331, 332].

Marrow Relapse

Approximately 80–90% of children experiencing a marrow relapse will achieve a second complete remission, often with agents similar to those used at the time of initial diagnosis [333, 334]; second complete remission rates are lower in those relapsing early and/or with T-ALL [328, 333, 335, 336]. Patients with persistent morphologic disease at the end of the first month of reinduction have a very poor prognosis, even if they subsequently achieve a second remission [337].

For B-ALL patients with early marrow relapses (defined as those occurring earlier than 30–36 months from initial diagnosis), allogeneic hematopoietic stem cell transplant (HSCT) has consistently been shown to be superior to chemotherapy-only approaches, although even with HSCT, long-term survival rates are less than 50% [338–340]. Patients with T-ALL, regardless of timing of relapse, are also generally treated with allogeneic HSCT because of poor survival rates with chemotherapy-only salvage therapy [341].

For B-ALL patients with a late marrow relapse, a chemotherapy-only approach leads to survival rates of approximately 50%, and it not clear that allogeneic HSCT is associated with superior outcome [328, 329, 338, 342]. End-reinduction minimal residual disease (MRD) levels strongly predict the prognosis of late-relapsing patients treated with chemotherapy only [331, 332]. Those with low MRD at this time point fare relatively well (event-free survival rates exceeding 70%), while those with higher MRD levels have a significantly greater risk of subsequent relapse [331, 332]. In one study, allogeneic stem cell transplant was associated with an improved outcome (compared to historic controls treated with chemotherapy only) in late-relapsing patients with high MRD levels at the end of reinduction [343].

For relapsed ALL patients who proceed to allogeneic HSCT, the components of the preparative regimen appear to impact the outcome; several studies have shown that regimens that include total body irradiation (TBI) are associated with better outcomes than those that do not [338, 344]. MRD levels at the time of transplant are also highly prognostic; patients with MRD-detectable disease just prior to HSCT fare worse than those with non-detectable levels [345–348].

Isolated Extramedullary Relapses

Isolated extramedullary relapses occur in fewer than 5% of patients. Using sensitive molecular techniques, submicroscopic marrow disease can be demonstrated in most children at the time of an isolated extramedullary relapse [349]. Thus, successful treatment strategies must address both the local site of relapse and submicroscopic systemic disease.

For patients with isolated CNS relapses, therapy typically involves intensive systemic chemotherapy with cranial radiation [350–352]. As with marrow relapses, patients experiencing late isolated CNS relapses have a better prognosis than those whose relapses occur earlier [329, 351–353], although a different cutoff is used to distinguish early versus late because of the overall more favorable outcome associated with extramedullary relapses. On two consecutive clinical trials conducted by the Pediatric Oncology Group, children with B-ALL and a late isolated CNS relapse (defined as initial remission duration of at least 18 months) had EFS rates exceeding 75% when treated with intensive chemotherapy and delayed cranial radiation, while patients with earlier relapses fared less well [351, 352]. It is not clear that allogeneic HSCT is associated with a survival advantage for patients with an isolated CNS relapse, even those with early relapses [354, 355]. Patients whose initial therapy included cranial radiation may have a worse prognosis after an isolated CNS relapse than previously unirradiated patients [350].

Isolated testicular relapses are uncommon, occurring in less than 1% of boys with ALL [239, 240]. For boys with isolated testicular relapses, systemic chemotherapy and testicular radiation and/or orchiectomy have resulted in prolonged second remissions in more than 80% of patients with late-occurring relapses [356, 357], with worse outcomes reported for those with earlier relapses [356, 358].

Future Directions

The improvement in cure rates for childhood ALL over the last several decades is due to the development of effective multiagent chemotherapy regimens, enhanced supportive

care, successful implementation of risk-adapted therapy, and advances in the understanding of disease biology. Despite this remarkable progress, some leukemia subtypes still respond poorly, and for patients who are successfully treated, therapy remains, to the large part, nonspecific and associated with multiple acute and long-term toxicities. Thus, there remains a need to further refine risk stratification and to develop more effective and potentially less toxic therapies.

Investigations of the genomic landscape of ALL have identified biologically distinctive subsets that may supplement or replace the currently applied clinical risk factors in order to identify those patients at highest risk of treatment failure [27, 64, 103, 104, 160, 359]. Druggable molecular lesions identified in various ALL subsets may lead to incorporation of targeted therapies, such as ABL-kinase and/or JAK-STAT inhibitors for *BCR-ABL1*-like ALL and epigenetic-modifying agents for *KMT2A*-rearranged ALL [109, 360]. In addition, research focused on germline genetic variation and pharmacogenomics may identify patient-related factors that affect the outcome and vulnerability to treatment-related toxicities, leading to more individualized therapy [361, 362]. The use of more sensitive molecular measures to assess minimal residual disease levels may enhance evaluation of early response [192, 348].

Novel therapies with the potential to improve outcomes include immunotherapeutic approaches. Although varying in their mechanisms of action, all of these approaches target cell surface antigens expressed on lymphoblasts, including CD19 and CD22 (nearly universally expressed in B-ALL), and thus represent a more targeted treatment strategy than current, nonspecific cytotoxic chemotherapy agents. Rituximab (anti-CD20 monoclonal antibody) has demonstrated benefit in adults with newly diagnosed CD20-positive ALL when given in combination with chemotherapy [363], and administration of single-agent inotuzumab ozogamycin, a recombinant immunotoxin consisting of humanized anti-CD22 antibody linked to calicheamicin, led to high complete remission rates in relapsed, refractory adult B-ALL patients [364]. Blinatumomab, a bi-specific (anti-CD3-anti-CD19 antibody) T-cell engager, has demonstrated single-agent activity in adult and pediatric relapsed B-ALL patients, and also has been shown to reduce minimal residual disease in those who had already achieved complete remission [365–367].

Cellular therapy using genetically engineered autologous chimeric antigen receptor (CAR) T-cells is an emerging and very promising immunotherapeutic approach. CAR T-cell therapy involves collection of autologous T-cells from patients, ex vivo genetic engineering to induce expression of ALL-specific chimeric antigen receptors along with additional co-stimulatory domains, and then reinfusion into the patient, typically after a chemotherapy preparative regimen [368, 369]. Phase 1 and 2 trials of CD19-directed CAR T-cells in relapsed/refractory pediatric and adult B-ALL patients have demonstrated high response rates, with some patients achieving sustained remissions without further treatment [369–371]. While there are many unanswered questions regarding CAR T-cells (duration of response, mechanisms of resistance, prevention of acute toxicities, potential long-term sequelae), the promising early outcome results highlight the potential of this treatment approach to transform therapy for relapsed and high-risk newly diagnosed ALL patients.

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18 Diagnosis and Treatment of Adult Acute Lymphoblastic Leukemia

Nicola Gökbuget and Dieter Hoelzer

Introduction

Acute lymphoblastic leukemia (ALL) is the most frequent neoplastic disease in childhood and accounts for about 20% of the acute leukemias in adults. An early peak of ALL incidence occurs at the age of 4–5 years and the overall incidence is 1.4 with a male predominance. It is relatively infrequent in younger adults but the incidence seems to increase in adults aged over 50 (Chap. 13). The median age of patients in most adult ALL studies ranges from 25 to 39 years.

In the past decades substantial progress has been made in the treatment of ALL mainly based on intensified chemotherapy regimens adopted from pediatric protocols, stem cell transplantation, risk- and subgroup-adjusted therapy, and improved supportive care. With contemporary regimens complete remission (CR) rates of 90% and cure rates of 40–70% depending on age can be achieved [1]. More recently individualized treatment according to minimal residual disease (MRD) has been implemented into treatment protocols. Targeted therapy particularly with tyrosine kinase inhibitors in Ph/BCR–ABL-positive ALL but also antibody therapy has contributed to further improved outcome in subgroups of ALL.

Clinical Features

The clinical presentation of adult ALL is almost always acute and the patient usually has symptoms of only a few weeks' duration. Patients show a rapid decline in general condition and feel generally ill. In rare cases, ALL may also develop from a transient preceding pancytopenia.

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Most of the symptoms of adult patients with ALL are nonspecific and are not usually severe. The patients may complain of progressive malaise with lethargy, fatigue, and occasionally weight loss. They may also complain of fever and night sweats in the absence of clinical infection. In older patients complications of anemia such as dyspnea, angina, and dizziness may dominate the clinical picture. Adult ALL patients may have minor arthralgias and bone pain but much less frequently than in children. One-third of the patients have infections or fever. As infections, hemorrhages are also less frequent than in acute myeloid leukemia (AML). There may be a history of some easy bruising and mucosal hemorrhage. In a series of 1273 consecutive ALL patients (15–65 years) treated in the German Multicenter Trials for Adult ALL (GMALL) one-third had some signs of minor bleeding tendency such as petechiae. Fever or infections had the same frequency. A few patients may present with neurological symptoms such as headache, alteration of mental function, or cranial nerve palsies due to leukemic infiltration.

An outline of the clinical approach to a patient with adult ALL is given in Table 18.1. Physical examination will show some degree of organomegaly in most adult patients with ALL: lymphadenopathy, usually cervical, is present in about half of the patients (57%) and palpable splenomegaly (56%) or hepatomegaly (47%) also in about half. A thymic mass can be found from chest roentgenograms or computer tomograms in 15% of all adult ALL patients. The majority of these patients have a T-cell ALL, but patients with other subtypes may occasionally also present with a mediastinal mass. Massive thymic enlargement can cause dyspnea, especially when associated with large pleural effusions. Some patients with mediastinal enlargement have pericardial effusions as well.

Presentation with clinically detectable signs related to leukemic infiltration of the central nervous system (CNS) occurs in about 5–10% of adult ALL patients [2]. Risk factors for CNS involvement include a high initial white blood cell (WBC) count, T-cell phenotype, and L3 or Burkitt's morphology. CNS involvement may manifest as raised intracranial pressure with headache and papilledema without

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Table 18.1 Adult ALL: clinical approach and laboratory investigation

Complete medical history, including
• Past medical history, especially heart, lung, liver, or renal disease, and diabetes mellitus (comorbidity score)
• Family history
• Occupational history
Complete physical examination, with special attention to lymphadenopathy and hepatosplenomegaly
• Temperature
• Potential sites of infection including lungs, oropharynx, and perineum
• Signs of abnormal hemorrhage
• Optic fundi
• Full neurological examination including the cranial and peripheral nerves
Diagnostic hematological studies
• Full blood examination including hemoglobin, platelet count, and white blood cell count (total and differential)
• Bone marrow aspirate and trephine biopsy
• Bone marrow cytology
• Immunological markers: T-cell, B-cell, immunological subtypes
• Cytogenetic analysis
• Molecular analysis, for prognostic markers, therapy targets, and MRD markers
Biochemical studies
• Including renal and hepatic function, serum uric acid, serum electrolytes including calcium and phosphate, blood glucose, and serum LDH
Coagulation studies
• Including prothrombin ratio, partial thromboplastin time, fibrinogen, and ATIII
Cardiac assessment
• Including electrocardiograph, echocardiogram, and other noninvasive tests of myocardial function if indicated
Chest roentgenogram: posterior–anterior and lateral
Computer tomograph, if mediastinal lymph nodes, tumor or abdominal masses are suspected
Serological studies
• ABO and Rh blood group
• HLA typing
Microbiological studies
• Culture from any infected site or lesion
• Surveillance cultures
• Serum for antibody titers, CMV, EBV, HIV, candida, aspergillosis
CSF examination
• Examination for cell count and cytocentrifuge preparation for morphology and, if necessary, immunophenotyping
Pregnancy test
Information about fertility preservation

HLA human leukocyte antigen; *PA* posterior–anterior; *CMV* cytomegalovirus; *EBV* Epstein–Barr virus; *HIV* human immunodeficiency virus

focal neurological signs or, rarely, as cranial nerve palsies, the sixth and seventh cranial nerves being most frequently involved. Careful examination of the ocular fundus must be made for leukemic infiltration as well as for hemorrhages due to thrombocytopenia.

Virtually any organ can be involved by infiltration of leukemic cells. The presence of bone lesions could be found in the earlier mentioned ALL series in only 1.2%. Also the initial involvement of the testis was very rare (0.3%). Other leukemic infiltrations were observed in the retina (0.9%), skin (0.9%), tonsils (0.5%), lung (0.5%), and kidney (0.5%). These organ manifestations present a typical clinical pattern of non-Hodgkin's lymphoma (NHL). They occur more frequently in mature B-cell ALL (32%) and T-ALL.

Diagnostic Procedures

The diagnosis of ALL is made by examination of the peripheral blood and bone marrow. Other investigations also need to be performed to further categorize and subclassify the disease and in preparation for therapy. These include cytochemical stains, immunological markers, cytogenetic analysis, and molecular genetic methods.

Peripheral Blood

Peripheral blood examination characteristically shows anemia, thrombocytopenia, and neutropenia (Table 18.2) although the total white blood cell count (WBC) is variable. The reduction in the level of hemoglobin is mild to moderate, but nearly one-third of the patients have a hemoglobin level below 8 g/dL. Although clinical bleeding due to thrombocytopenia is not very common, about half of the patients have a platelet count below 50×10^9/L. The proportion (30%) of adult ALL patients having had some history of hemorrhage corresponds well with the 30% of patients with a platelet count below 25×10^9/L. The proportion of patients with a granulocyte count below 0.5×10^9/L, usually associated with high risk of infection, was only one-fifth in this series. Only a small minority of patients had clotting defects and of these 5% had an initially decreased fibrinogen level, which might be of relevance if an immediate l-asparaginase treatment is anticipated. The WBC was reduced in 27%, and normal or modestly elevated in 60%, and 16% had a marked leukocytosis (WBC count > 100×10^9/L) at presentation. However, even in the cases where the WBC was reduced or normal, characteristic lymphoblasts could be identified on a well-stained blood smear in more than 90% (Table 18.2).

Table 18.2 Laboratory findings at diagnosis of adult ALL

	1273 patients (%)
Total leukocytes (×10^9/L)	
<5	27
5–10	14
10–50	31
50–100	12
>100	16
Granulocytes (×10^9/L)	
<0.5	22
0.5–1.0	14
1.0–1.5	9
>1.5	55
Hemoglobin (g/dL)	
<6	8
6–8	20
8–10	26
10–12	24
>12	22
Thrombocytes (×10^9/L)	
<25	30
25–50	22
50–150	33
>150	16
Fibrinogen (mg/dL)	
<100	5
>100	95
Leukemic blast cells in PB	
Present	92
Absent	8
Leukemic blast cells in BM	
<50%	4
51–90%	25
>90%	71

Data from unpublished GMALL studies

Bone Marrow

Bone marrow examination provides further material for diagnostic assessment including morphology, cytochemical stains, immunological markers, and cytogenetic and molecular analysis. Smears of the bone marrow aspirate show markedly hypercellular particles. The majority of cells are leukemic lymphoblasts. A total of 97% of the adult ALL patients had a bone marrow infiltration with leukemic lymphoblasts above 50% (Table 18.2). The normal hematopoietic elements are greatly reduced or absent but, in contrast to AML, they have essentially normal morphology. The trephine biopsy of the bone marrow will further demonstrate marked hypercellularity with replacement of fat spaces and normal marrow elements by infiltration with leukemic cells. A slight increase in marrow reticulin is seen in a small proportion of patients with ALL but much less commonly than with AML. If an adequate bone marrow aspiration is available, it remains open whether an additional biopsy should be done. In our hands a biopsy was necessary when aspiration was not possible due to heavily packed leukemic cells or increased reticulin fibers.

Laboratory Investigations

The laboratory investigations that should be performed at the time of diagnosis (Table 18.1) will serve as a baseline for subsequent studies during the induction period, and may also document metabolic abnormalities that require correction before the start of treatment or modification of drug dosage. Renal impairment, hyperuricemia, and electrolyte imbalance should be corrected if possible before treatment is begun. Serum lactic dehydrogenase (LDH) levels are markedly elevated in most patients with ALL. A full hemostatic profile should be performed to detect the very occasional adult ALL patients with disseminated intravascular coagulation or with an incidental clotting abnormality related to preexisting liver disease or liver infiltration. Besides cultures from any clinically infected site, surveillance cultures from the nose, throat, axillae, groin, vagina, perianal area, and sputum and urine are taken to detect clinically occult infection and to provide useful information about microbiological etiology if septicemia or severe infection subsequently develops. In patients with a past medical history of heart disease and in older patients where treatment with an anthracycline is anticipated, an echocardiogram with myocardial function, including the ejection fraction, should be carried out.

Cerebrospinal Fluid

The examination of the cerebrospinal fluid (CSF) is an essential diagnostic procedure in ALL to exclude or confirm initial CNS disease. There are different opinions as to when the first lumbar puncture should be done. Early recognition of CNS disease is clinically important because more aggressive CNS therapy is required for such patients. Therefore a diagnostic puncture should be done if possible before initiation of systemic chemotherapy. This procedure is restricted to patients with an adequate platelet count which can be achieved by platelet transfusion (>20 × 10^9/L), an absence of manifest clinical hemorrhages, and without a high WBC. For safety reasons patients should receive intrathecal methotrexate at

the first lumbar puncture. Clearly this procedure necessitates an atraumatic lumbar puncture and should only be performed by experienced physicians since in childhood ALL blood contamination of the CSF was associated with a higher relapse risk. In pediatric studies nowadays CNS disease at diagnosis is classified into four groups: CNS1 (no blasts in CSF), CNS2 (<5 WBC/μL with blasts), CNS3 (≥5 WBC/μL with blasts), and TLP+ (traumatic lumbar puncture with ≥10 RBC/μL with blasts) [2]. CNS involvement in adult ALL is generally defined as CNS3 or the presence of signs of CNS involvement in CT or MRT or neurological symptoms not otherwise explainable, e.g., cranial nerve palsies.

Differential Diagnosis

Difficulty is rarely experienced in establishing the diagnosis of ALL. Viral infection may cause lymphadenopathy and hepatosplenomegaly with lymphocytosis in the blood and bone marrow and, although the distinction can usually be made on clinical and morphological grounds, the results of viral antibody titers, lymphocyte surface markers, and cytogenetic analyses may be required. The leukemic phase of non-Hodgkin's lymphoma can mostly be recognized by clinical and morphological features, by the type and pattern of immunological cell surface markers, and by the degree and distribution of bone marrow infiltration. In the rare cases with a low bone marrow infiltration an arbitrary distinction between ALL and lymphoblastic lymphoma is usually chosen according to the degree of infiltration, above or below 25%. With more advanced immunological marker application, mixed leukemias having myeloid as well as lymphoid surface markers are diagnosed, which might be allocated to a treatment strategy for either ALL or AML; recent data show more favorable outcomes with ALL regimens for biphenotypic leukemias [3]. They have to be distinguished from cases with ALL and coexpression of myeloid markers, which is rather frequent in immature subtypes such as pro-B-ALL or early T-ALL. These patients are treated with ALL strategies.

Occasionally, difficulties can occur in distinguishing Ph/BCR–ABL-positive ALL from primary lymphoid blast crisis of CML. Sometimes final diagnosis can be done only after treatment initiation. In ALL patients achieving complete clinical remission (CR), the peripheral blood count shows normal values, whereas CML cases may revert to a chronic phase with pathological left shift.

Classification

There is a wide heterogeneity within ALL. Therefore accurate morphological classification, determination of the immunological phenotype, and cytogenetic and molecular genetic analysis, which are of prognostic and therapeutic relevance, should be performed in every case of ALL, including older ALL patients. In addition, in all patients, material from the time point of diagnosis should be sent to a reference laboratory in order to identify individual markers for detection of minimal residual disease.

Morphology and Cytochemistry

Bone marrow aspirates and blood smears are stained with Wright's or Wright's-Giemsa stain and the blast cells may be classified according to the French–American–British (FAB) classification. Clinical relevance of FAB subtypes is limited to the detection of the L3 FAB subtype which is characteristic for mature B-ALL. This subtype is important to identify since different treatment approaches are used. In the new WHO classification ALL is classified together with lymphoblastic lymphoma into B-precursor lymphoblastic leukemia/lymphoma, T-precursor lymphoblastic leukemia/lymphoma, and Burkitt's leukemia/lymphoma [4]. The further subclassification is of less relevance for management of adult ALL.

The cytochemical stains to discriminate between AML and ALL are Sudan black, myeloperoxidase, and chloracetate or nonspecific esterase. These reactions are negative in ALL, negativity being usually defined as less than 3% of leukemic blast cells positive. Cytochemical stains to confirm ALL are periodic acid-Schiff (PAS) and acid phosphatase. The PAS stain will show coarse granules or block positivity in at least some cells of most patients with adult ALL of the L1 or L2 type, the incidence of positivity being approximately 60–70% in both groups. The acid phosphatase reaction is positive in 20–30% of all ALL being more specific for T-ALL. About 70% of patients with T-ALL will show strong and localized paranuclear staining with acid phosphatase. PAS or acid phosphatase reactivity is, however, not restricted to ALL and since it can be positive in some cases (M5) of AML the additional reactions for peroxidase and acetate esterase must be negative.

Immunophenotyping

The main aim of immunophenotyping of leukemic blast cells is to distinguish between AML and within the ALL between B- or T-lineage ALL by using monoclonal antibodies to pan-B (CD19), pan-T (CD7), and pan-myeloid surface antigens (CD13, CD33, CDw65). To detect early lymphoid or myeloid differentiation, lineage-specific markers which are first exhibited in the cytoplasm (cy) of B-cell (cyCD22), T-cell (cyCD3), and myeloid precursor cells (myeloperoxidase) are used. To define further maturational stages within the B- and T-cell lineages, markers more specific for

Table 18.3 Immunological, morphological, cytogenetic, and molecular characterization of ALL[a]

Subtypes	Marker	Incidence[a] (%)	FAB subtype	Frequent cytogenetic aberrations	Fusion transcripts and mutations
B-lineage ALL	**HLA-DR⁺, TdT⁺, CD19⁺and/ or CD79a⁺and/or CD22⁺**	**76**			
Pro B-ALL	No additional differentiation markers	12	L1, L2	t(4;11)(q21;q23)	70% ALL1-AF4 (20% Flt3 in MLL+)
Common ALL	**CD10+**	49	L1, L2	t(9;22)(q34;q11) del(6q)	33% BCR–ABL (30–50% in c/preB) 4% t(1;19)/PBX-E2A 10–20% BCR-ABL-like[c]
Pre-B-ALL	CD10±, **cyIg+**	11	L1, L2	t(9;22)(q34;q11) t(1;19)(q23;p13)	
Mature B-ALL	CD10 ±, sIg+	4	L3	t(8;14)(q24;q32) t(2;8)(p12;q24) t(8;22)(q24;q11)	
T-lineage ALL	**cyCD3 orsCD3**	**23**			
Early T-ALL Cortical T-ALL Mature T-ALL	No additional differentiation markers, mostly CD2− **CD1a+**, sCD3 ± sCD3+, **CD1a−**	6 12 5	L1, L2	t/del(9p) t(10;14)(q24;q11) t(11;14)(p13;q11)	5% NUP214-ABL1[b] 30% TLX1[b] >60% Notch1[b]

[a]*N* = 946 adult ALL patients [7]
[b][8]
[c][9]

particular maturational stages are used: for B-lineage CD20, cy immunoglobulin μ heavy chain (cyIgM), and surface immunoglobulin (sIg), and for T-lineage CD1, CD2, CD4, CD8, and surface sCD3. The maturation stages are not identified by the presence or absence of a single antigen but by a pattern of antigen expression. One widely used classification system for immunologic subtypes in ALL has been proposed by the EGIL group [5]. More recently early T-precursor ALL has been described as a specific subtype of T-ALL, characterized by a unique gene expression and surface marker profile [6]. For further details on immunobiology of ALL refer to Chap. 15.

With the availability of more specific monoclonal antibodies 98–99% of the acute leukemias can now be reliably classified by immunological marker analysis. In addition, ALL can be subdivided according to various maturational stages of B- or T-lineage, whereby it is assumed that they are in differentiation arrest corresponding to normal maturational stages. Immunological classification of ALL subtypes is summarized in Table 18.3.

B-Lineage ALL

With the analysis of CD10 (the common ALL antigen), cyIgM, and sIg, the B-lineage ALL can be subdivided into three subgroups of B-cell-precursor ALL and mature B-ALL. Virtually all B-precursor ALLs are positive for HLA-DR and TdT. Pro-B-ALL (also termed pre-pre-B-ALL, null-ALL, CD10-negative ALL) is the most immature subtype of the B-ALL lineage. This subtype is characterized by the expression of CD19, cyCD22, and mostly CD24, while CD10, cyIgM, and SIg are negative. Common ALL, the major immunological subtype in childhood as well as in adult ALL, is characterized by the expression of CD10 in combination with CD19, cy or sCD22, and CD24. Common ALL blast cells do not carry markers of relatively mature B-cells such as cyIgM or sIg. Pre-B-ALL is characterized by the expression of cyIgM, being negative in common ALL but otherwise identical with all other markers, such as CD19, cy or sCD22, CD24, and only very rarely CD10 may be absent in this subtype. In most adult clinical studies pre-B-ALL is included in the common ALL category. In most studies common ALL is defined by surface antigen expression of CD10 on 20% or more of leukemic cells and the diagnosis of pre-B-ALL by cyIgM in 10% or more of blast cells.

Leukemic blast cells in mature B-ALL, also termed Burkitt's leukemia, express sIg and B-cell antigens including CD19, CD20, CD22, CD24, and usually CD10. In contrast to the B-precursor ALLs, leukemic cells in B-ALL are mostly negative for TdT. Most B-ALLs can be identified morphologically as L3 FAB subtype.

T-Lineage ALL

Early T-ALL is characterized by the expression of cyCD3 with no additional differentiation markers. CD2 is generally negative. In cases positive for CD2 but negative for CD4, CD8, sCD3, and CD1a, an early T-ALL is present as well. Cortical T-ALL, also referred to as thymic T-ALL, is characterized by the expression of CD1 in combination with CD7, CD5, and CD2, and sometimes also sCD3, CD4, and CD8. Mature T-ALL is characterized by positivity for sCD3, CD7,

CD5, and CD2 while CD1 is negative. CD4 and CD8 are present in most cases. In the newly described entity of early T-precursor ALL (ETP) by definition, blasts in ETP ALL express CD7 but lack CD1a and CD8, and are positive for one or more of the myeloid/stem cell markers [4].

Myeloid Antigen-Positive ALL (My + ALL)

Immunophenotyping has shown the existence of acute leukemia cases in which the blast cells express markers supposedly specific for or associated with another cell lineage. The myeloid-antigen-associated monoclonal antibodies that are used for the detection of My + ALL are CD13, CD14, CD15, CD33, and CDw65. The reported frequencies of My + ALL differ widely, ranging from 5 to 46% depending on the definition, and an approximate figure for adult My + ALL may be 18%. Commonly a case is considered as My + ALL if 20% or more of the blast cells are reactive with the myeloid-lineage-associated monoclonal antibodies. The expression of myeloid antigens is associated with certain subtypes of ALL such as pro B-ALL or early T-ALL. Myeloid coexpression should be differentiated from biphenotypic leukemia. The EGIL group has proposed a score for identification of biphenotypic acute leukemia [5].

Table 18.4 Characteristics of immunological subtypes of adult ALL[a]

Subgroup	Clinical/laboratory characteristics	Relapse kinetics and localization
B-lineage		
Pro-B-ALL	– t(4;11)/ALL1-AF4 (70%) – High WBC (>100/mL) (26%) – Frequent myeloid coexpression (>50%)	– Mainly BM (>90%)
c-ALL/ pre-B-ALL	– Higher age (24% > 50 year) – Ph/BCR–ABL (40–50%) – m-BCR (70%), M-BCR (30%)	– Mainly BM (>90%) – Prolonged relapse kinetics (up to 5–7 year)
B-ALL	– Higher age (27% > 50 year) – Frequent organ involvement (32%) – Frequent CNS involvement (13%)	– Frequent CNS (10%) – Short relapse kinetics (up to 1–1½ years)
T-lineage		
	– Younger age (90% < 50 year) – Frequent mediastinal tumors (60%) – Frequent CNS involvement (8%) – High WBC (>50/mL) (46%)	– Frequent CNS (10%)/ extramedullary (6%) – Intermediate relapse kinetics (up to 3–4 years)

[a]Data based on German multicenter trials of adult ALL

Frequency and Clinical Features of Immunological Subtypes

The frequency of immunological subtypes in adult ALL shows distinct differences from that in childhood ALL. Approximately 84% of the children have a B-precursor ALL and common ALL is, with 63%, the most frequent subtype, whereas this subtype is only observed in one-half of adult ALL patients. There is a significantly higher proportion of the pro-B-ALL, with 12% in adults compared to only 5% in children. Mature B-ALL is rare in both childhood and adult ALL. Adult T-ALL, with 23%, has a twofold higher incidence in adults than the 13% observed in children. As in the B-lineage the most immature form, pre-T-ALL, has a higher incidence in adults compared to children.

The clinical features of the immunological subtypes of ALL are quite distinct. The immature pro-B-ALL has a peak in infants less than 1 year old and is associated with high WBC, massive hepatosplenomegaly, CNS disease, and myeloid coexpression, and approximately 70% of the patients show t(4;11). Nearly one-half of the patients with c-ALL or pre-B-ALL show the translocation t(9;22) and the incidence increases with age. Mature B-ALL is characterized by frequent abdominal tumor masses, often organ involvement, an increased incidence of CNS leukemia, and a male preponderance. T-ALL is associated with mediastinal masses in nearly half of the patients, occasionally associated with pleural and pericardial effusions, an increased incidence of organomegaly, a higher incidence of CNS disease, a high WBC count, and male prevalence. The major clinical differences between immunological subtypes of adult ALL are summarized in Table 18.4.

Cytogenetics and Molecular Genetics

Cytogenetic and molecular genetics abnormalities are independent prognostic variables for predicting the outcome of adult ALL (Chap. 16). In several multicenter studies, clonal chromosomal aberrations could be detected in approximately 62–85% of adult ALL patients. The most frequent numerical chromosomal aberrations are hypodiploid karyotype with less than 46 chromosomes (4–8%), hyperdiploid karyotype with 47–50 chromosomes (7–15%), or greater than 50 chromosomes (7–8%). The most frequent structural aberration is the translocation t(9;22)/Philadelphia chromosome (P+ ALL). Other translocations occur less frequently and are mostly associated with distinct immunological subtypes such as t(4;11) (3–4%) in pro-B-ALL, t(8;14) (5%) in mature B-ALL, t(1;19) (2–3%) in pre-B-ALL, and t(10;14) (3%), 9p– (5–15%), 6q– (4–6%), and 12p aberrations (4–5%) mainly in T-ALL.

The Ph chromosome t(9;22)(q34;q11) results from a translocation involving the breakpoint cluster region of the BCR gene on chromosome 22 and the ABL gene on chromosome 9. One-third of adult ALL patients with a Ph chromosome show M-BCR rearrangements (resulting in a 210 kDa protein), similar to patients with CML, whereas two-thirds have m-BCR rearrangements (resulting in a 190 kDa protein).

Currently a new subgroup with prognostic and therapeutic relevance is discussed. The "Ph-like" or "BCR-ABL-like" ALL is characterized by a gene expression profile similar to Ph-positive ALL. A part of the patients also show translocations or mutations of ABL or JAK genes, which can potentially be targeted by tyrosine kinase inhibitors [9, 10]. Patients with this subtype of ALL show an inferior prognosis in childhood and adults with lower rates of molecular responses [9–11]. A prospective and validated diagnostic identification of Ph-like ALL is currently not possible but identification of targetable lesions may be helpful in individual patients with refractory disease.

The most frequent form of 11q23 abnormalities in ALL is t(4;11)(q21;q23). The translocation is frequently detected in infant leukemia and in patients with the pro-B-ALL subtype (CD10 negative). The overall incidence in adults is approximately 5%. Typical molecular aberrations in ALL with associated cytogenetic translocations and immunological subtypes are summarized in Table 18.3.

Blasts cells with a low hypodiploid karyotype represent another high-risk entity which is frequently associated with TP53 mutations [12].

Overall the observed incidence of the majority of cytogenetic aberrations is very low and therefore the options of correlation to clinical outcome and even more therapeutic consequences are limited. The most relevant markers can be identified by molecular analysis as well. Nevertheless, cytogenetic analysis is still recommended as a routine diagnostic method in ALL. It is highly recommended to store biomaterials from each ALL patient for potential future analyses, e.g., focused on targetable lesions.

Detection of Minimal Residual Disease

Conventional microscopic evaluation of bone marrow smears has a detection limit of 5%. With methods for detection of MRD, residual blast cells can be detected and measured quantitatively below this level with a sensitivity of 10^{-4}–10^{-6}. With these methods individual follow-up analyses can be performed in patients with clinical and morphological CR. ALL is an "ideal" disease for detection of MRD since more than 90% of the patients show individual clonal markers. Most experience has been accumulated with MRD detection by flow cytometry and PCR (overview in [13]).

MRD detection by flow cytometry targets individual leukemia-specific combinations of surface antigens and reaches a sensitivity of 10^{-4}. PCR detection may target the expression of leukemia-specific fusion genes such as BCR–ABL, which may be detected in 30–40 of adult ALL cases. A more widespread applicability is reached with detection of clonal rearrangements of immunoglobulin heavy-chain (IgH) or T-cell receptor (TCR- β, −δ, −γ) gene rearrangements. This method reaches a sensitivity of 10^{-4}–10^{-6} and combinations of two or more target structures can be identified in more than 80% of ALL patients. For this method the best level of standardization has been reached regarding methodology and reporting and interpretation of results for clinical trials [14]. MRD detection with any method should be restricted to experienced laboratories, which participate in quality control rounds taking place on an international level.

Markers for MRD detection have to be established at the time of first diagnosis. Therefore diagnostic material has to be provided to a specialized laboratory.

Supportive Care

The management of adult patients undergoing induction therapy for ALL requires intensive treatment of initial complications and supportive care to prevent and manage the infectious, hemorrhagic, metabolic, and psychological problems that may arise.

Metabolic Abnormalities

A few general measures can be started at once. Sufficient fluid intake to guarantee urine production of at least 100 mL/h throughout induction therapy reduces the risk of uric acid formation. This may require parenteral fluid administration when the patient's oral intake is inadequate because of nausea or difficulty in swallowing. If the venous system does not offer an easy approach, access by catheter or port is advantageous when anticipating a longer period of induction therapy or when part of the therapy will be carried out on an outpatient basis.

Hyperuricemia is frequently present at diagnosis; it may worsen following the initiation of chemotherapy and, if not treated, can lead to renal failure. Adequate doses of allopurinol (300–600 mg/day) should be given and the urine alkalinized before chemotherapy. Allopurinol has to be reduced when 6-mercaptopurine is given. In patients with high risk of tumor lysis, uratoxidase may be used for prevention of hyperurikemia.

In patients presenting with renal impairment, an attempt must be made to reestablish renal function before chemotherapy is started. Renal failure is often observed in patients

with Burkitt's lymphoma or B-ALL with abdominal tumor masses and can be resolved by a gentle pretreatment with cyclophosphamide (C) combined with dexamethasone (P) or dexamethasone (DX) or steroids alone.

The acute tumor lysis syndrome is most frequently seen in patients with B-ALL or T-ALL but may also occur in other subtypes with high WBC or large tumor mass. Massive and rapid tumor cell lysis leads to hyperkalemia, hyperphosphatemia, hyperuricemia, and hypocalcemia, which can largely be prevented by the C + P/DX treatment.

Infections

Approximately one-third of adult ALL patients present with infections at diagnosis. Fever or infection at the time of admission is mainly associated with severe granulocytopenia, especially if the granulocyte count is less than 5×10^9/L but may also be due to immunological deficiency (e.g., CD4 lymphopenia) or mucosal lesions. Combination chemotherapy causes additional hematological toxicity and at least 50% of adults undergoing induction treatment will experience severe or life-threatening infections. The incidence of infections with gram-positive bacteria has increased—especially those due to more frequent use of indwelling catheters. Fungal infections also occur more frequently.

Much attention has been paid to prophylactic measures against infection. They include oral hygiene using antiseptic soaps and mouthwashes and disinfection of the anogenital region. Other precautions include reverse protective isolation and air filtration, if available, which can reduce especially the risk of Aspergillus infections. Simple precautions that can always be carried out are no live plants in the room, no humidifiers, no i.m./s.c. injections if avoidable, no uncooked vegetables, no unpeeled fruits, and no visitors having any kind of infection. Prevention and management of infections are discussed in detail in Chaps. 49 and 51.

Hematopoietic Growth Factors

The use of hematopoietic growth factors (HPGFs) such as colony-stimulating factor–granulocyte (G-CSF) is a valuable component of supportive therapy during the treatment of ALL. There is no indication that these CSFs stimulate leukemic cell growth in a clinically significant manner. The majority of clinical trials demonstrate that the prophylactic administration of G-CSF significantly accelerates neutrophil recovery and several prospective randomized studies also showed that this is associated with a substantially reduced incidence and duration of febrile neutropenia and of severe infections [15, 16]. The enhanced marrow recovery allows closer adherence to the dose and schedule of chemotherapeutic regimens.

The advantage of G-CSF administration is particularly evident in patients at high risk for prolonged granulocytopenia. Furthermore, scheduling appears to be important. When CSFs are first given at the end of a 4-week chemotherapy regimen, potential benefits are limited. Therefore it is noteworthy that G-CSF may be given in parallel with chemotherapy without aggravating the myelotoxicity of these specific regimens [15, 16] and that this scheduling is an important determinant of the clinical efficacy.

Hemorrhage

The thrombocytopenia present in one-third of the patients at diagnosis will worsen following chemotherapy, requiring transfusion of platelet concentrates. Platelet transfusions should be given for bleeding and to prevent bleeding when platelet counts are below 20×10^9/L especially during febrile periods, which interfere with platelet function. When a long induction period is anticipated and there is a likelihood that a patient will need frequent platelet transfusions it might be preferable to start with HLA-matched platelets immediately, if this is logistically possible (technical facilities, costs), to avoid refractoriness to random platelets. The issue of platelet transfusions is discussed in detail in Chap. 54.

L-Asparaginase treatment leads to a decrease in fibrinogen and ATIII and may thereby enhance the risk of thrombosis and bleeding. So far no standards have been defined for substitution of both factors although it is done in many trials.

Chemotherapy

The approach to therapy of adult ALL has evolved along similar lines to that successfully employed in childhood ALL. An induction therapy is followed by a postremission or consolidation therapy. Whereas the induction phase of therapy is usually well defined, the postremission therapy may consist of different consolidation cycles, including reinduction or stem cell transplantation. In addition there is a CNS prophylaxis throughout the whole therapy and maintenance treatment.

Traditionally successful treatment protocols for adult ALL are based on pediatric approaches. The overall outcome is evident from the published studies in younger ALL patients listed in Table 18.5. Complete remission (CR) rates in modern protocols reach 90% with approximately 5% early

Table 18.5 Results of recent trials with pediatric based regimens in adult ALL

Study	Year	*N*	Median age (range)	CR rate (%)	Survival (%)
Ribera et al.[a] [17]	2008	81	29 (15–30)[a]	98	69 (6 years)
Huguet et al. [18]	2009	225	31 (15–60)	93	60 (3 years)
Rijneveld et al. [19]	2011	54	26 (17–40)	91	72 (2 years)
Stock et al. [20]	2014	296	24 (17–39)	nr	78 (2 years)
Rytting et al. [21]	2014	84	21 (13–39)	94	74 (4 years)
De Angelo et al. [22]	2015	92	28 (18–50)	85	67 (4 years)

[a]Standard risk only

death during induction and 5% failure to achieve a remission. Overall survival and leukemia-free survival range between 40 and 70% with a large variability according to subgroups of ALL with around 30–50% survival for high risk, 40–70% for standard risk, 50–70% for Ph+ ALL, and 70–80% for mature B-ALL.

Initial Treatment

In patients with a large leukemic cell burden, that is, a high WBC and/or massive organomegaly, cell reduction with a cautious preinduction therapy is recommended. In patients with high WBC count (>100 × 10^9/L), where hyperviscosity due to leukostasis with cerebral impairment may occur, leukopheresis may be considered. However, such technical facilities may not be available and these patients can also be managed with a gentle prephase chemotherapy consisting of vincristine or cyclophosphamide and prednisone or dexamethasone in nearly all cases without complications. Prephase treatment is suitable anyway in order to stabilize the patients and complete all diagnostic procedures before the start of induction treatment.

Remission Induction

Standard induction therapy for ALL includes prednisone, vincristine, anthracyclines, mostly daunorubicin, and also L-asparaginase. Further drugs, such as cyclophosphamide, cytarabine (either conventional or high dose), mercaptopurine, and others, are added in many protocols, sometimes named as early intensification.

Steroids such as prednisone and prednisolone have been most frequently administered. Dexamethasone shows a higher antileukemic activity in vitro and a better penetration of the cerebrospinal fluid. Extensive use of DX without interruptions may, however, be associated with an increased risk of septicemias and fungal infections, which may be circumvented if treatment time is reduced.

The most frequently used anthracycline is *daunorubicin* (DNR). Several study groups have replaced the usual weekly applications, as in the BFM-based protocols, by higher doses of DNR (45–80 mg/m^2) on subsequent days. In one recent trial the intensified use of daunorubicin failed to improve response rates [23]. Thus it remains open whether intensified anthracyclines are beneficial for adult ALL at all, for all subgroups and age groups. Intensive anthracycline therapy may be associated with an increased induction mortality. Therefore, intensive supportive care and probably the use of growth factors are recommended with these types of protocols.

Asparaginase (A) does not affect the CR rate but improves LFS. If not used during induction therapy, it is often included as part of the consolidation treatment. Three different A preparations with significantly different half-lives are available: native *E. coli* A (1.2 days), Erwinia A (0.65 days), and pegylated *E. coli* A (PEG-L-A) (5.7 days). The availability may vary between different countries. In order to reach equal efficacy, the application schedule has to be adapted and is generally daily for Erwinia, every second day for *E. coli*, and 1–2 weeks for PEG-L-A. The latter asparaginase preparation has the advantage of less frequent administrations and more even activity distribution. In a considerable proportion of adult ALL patients A induces laboratory changes, e.g., coagulation disorders and liver transaminases with unclear clinical impact [24] and in fewer patients severe complications such as hepatopathies or pancreatitis. A-induced toxicities are not always predictable and may lead to treatment delays in individual patients. Consistent management of toxicities is essential [25]. On the other hand, ASP is recognized as an extremely important drug for the treatment of ALL due to its unique mechanism of action and resistance. Optimization of A therapy is therefore a major aim for management of adult ALL.

Definition of Complete Remission

Complete remission is defined as a state in which there is no clinical or laboratory manifestation of leukemia. The peripheral blood count and bone marrow appearances are within normal limits except for abnormalities attributable to chemotherapy; the marrow blast cell count is less than 5%; also examination of the CSF shows no blast cells. CR includes also the disappearance of organomegalies, but it should be noted that the persistence of splenomegaly is not always due to leukemic infiltration.

Table 18.6 Definition of response in ALL

Conventional definition		Evaluation of MRD	
Complete remission	• <5% blasts in bone marrow smear and • Regeneration of peripheral blood count and • Disappearance of all extramedullary manifestations	Molecular remission	Negative MRD status detected with a standardized method and a minimum sensitivity of 0.01%
		Molecular failure	Positive MRD-status above 0.01%
Relapse	• Detection of more than 5% blasts in bone marrow after prior achievement of CR[a] or • Reappearance of extramedullary manifestations	Molecular relapse	Increase of MRD above 0.01% after prior achievement of molecular CR

[a]In case of 5–10% blasts in regenerating marrow a repeated bone marrow assessment is recommended in order to distinguish blasts form hematogones

Definition of complete remission was recently extended by the definition of MRD response or molecular response. An international consensus workshop has defined technical prerequisites for MRD-based response evaluation mainly for PCR-based measurement of individual gene rearrangements. In patients with a marker with sensitivity of at least 10^{-4}, complete MRD response is defined as a negative status of MRD. MRD failure is defined as MRD level above 10^{-4} [14]. MRD response is strongly associated with prognosis. Therefore MRD-based response evaluation is a new endpoint for clinical trials (Table 18.6).

Failure of Induction Therapy

With current protocols failure rates after induction are generally around 10%. The rate of early death depends on age and ranges from <3% in adolescents to 20% in patients >60 years of age. The main cause of death in approximately two-thirds of the patients is infection, in part fungal infection. Beyond mortality also morbidity, e.g., due to extended cytopenias and subsequent infections such as fungal pneumonias, has to be considered which may compromise further treatment and dose intensity. The remaining nonresponders may achieve a partial remission or may be refractory to standard treatment. These patients have an extremely poor prognosis. They are therefore candidates for experimental treatment approaches or consideration for an SCT, even if not in CR but in good partial remission.

Consolidation Therapy

When in ALL CR is achieved, treatment has to be continued in order to eliminate residual leukemia after induction chemotherapy and thereby prevent relapse as well as emergence of drug-resistant cells because a high percentage of patients show MRD after induction therapy. Continuation or postremission therapy consists of intensification or consolidation and maintenance. Consolidation/intensification refers either to high-dose therapy, the use of multiple new agents, or readministration of the induction regimen (reinduction). SCT is also included in postremission therapy in many trials. In most studies that involve repeated consolidation cycles over the entire treatment period, it is difficult to analyze critically the effect of the different treatment phases on outcome.

Intensive consolidation is standard in the treatment of ALL although consolidation cycles in large studies are very variable and it is impossible to evaluate their individual efficacy. In general it seems that intensive application of high-dose methotrexate (HDMTX) is beneficial. Depending on age, in adults dosages are probably limited at 1.5–3 g/m^2 if given as 24-h infusion. Otherwise toxicities, particularly mucositis, may lead to subsequent treatment delays and decreased compliance. From pediatric ALL trials there is increasing evidence that intensified application of asparaginase leads to improved overall results. In adult ALL this approach appears to be useful particularly in consolidation, where less toxicity can be expected compared to induction. Several studies have also demonstrated that a reinduction improves outcome.

The most important feature of consolidation is probably to administer rotating cycles with short intervals. However after several consolidation cycles some adult patients tend to develop prolonged cytopenias, which lead to delays of subsequent chemotherapies. Therefore a balance between bone marrow toxic and less toxic cycles may be important. For future studies in adult ALL stricter adherence to protocols with fewer delays, dose reductions, and omissions would be an important contribution to therapeutic progress.

Maintenance

Maintenance up to a total treatment duration of 2½ years even after intensive induction and consolidation is still standard for adult ALL; all attempts to omit maintenance led to inferior outcome. MTX preferably given intravenously (i.v.) or orally and mercaptopurine (MP) given orally are the backbone of maintenance. It may be important to aim for

leukocyte counts below 3000/μL during maintenance [26]. Intensification cycles with vincristine and steroids did not provide additional benefit at least in pediatric trials using intensive reinduction [27]. Furthermore in adults prolonged steroid therapy may lead to an increase of late effects such as osteonecrosis. Randomized trials also failed to demonstrate an advantage of intensified maintenance with HD cycles although the compliance in these trials is unclear.

Adults often show poor compliance to intensive maintenance due to toxicities and moreover social reasons. Even for conventional maintenance compliance may be a problem. In Ph+ ALL maintenance with kinase inhibitors appears to be of utmost importance after chemotherapy as well as after stem cell transplantation. Overall, for further improvement of outcome the physicians' and patients' compliance to maintenance therapy seems to be essential.

Central Nervous System Therapy

Prophylactic CNS Therapy

Without some form of prophylactic CNS therapy, around 30% of adults with ALL will develop overt CNS leukemia [2]. Prophylactic CNS therapy in ALL is essential due to several reasons: CNS leukemia is more easily prevented than treated; once CNS leukemia has developed, it is generally followed by systemic relapse shortly after; and effective CNS prophylaxis also prevents systemic relapse.

Several treatment options are available for prevention of CNS relapse: intrathecal (i.th.) therapy, cranial irradiation (CRT), and systemic high-dose chemotherapy (overviews in Ref [2]). I.th. therapy is usually based on MTX as single drug but combinations with AC and/or steroids are used in some studies. The route of application is generally lumbar puncture. CRT (18–24 Gy in 12 fractions over 16 days) may be administered with or without parallel i.th. therapy. Systemic HD chemotherapy may comprise HdAC or HdMTX since both drugs reach cytotoxic drug levels in the CSF and showed effectivity in overt CNS leukemia.

Various combinations of these approaches have been used in adult ALL trials but the issue of CNS prophylaxis has not been addressed prospectively. For analysis of published trials, it has to be considered that most authors only report the frequency of isolated CNS relapses. However, in a significant proportion of adult ALL patients combined CNS and bone marrow relapses occur. Overall there is evidence that CNS relapse rates decrease with increasing intensity of prophylaxis and with the number of applied modalities.

The role of CNS irradiation in different subtypes of adult ALL remains to be determined. In pediatric patients considerable late effects of CNS irradiation are observed and effective CNS prophylaxis is achieved by high doses of systemic methotrexate treatment. In adults similar doses cannot be administered due to toxicities. Therefore the role of CNS irradiation may be different; in addition there are no reports on similar late effects compared to those observed in children.

In current trials with effective CNS prophylaxis the incidence of CNS relapse is below 5–10%. It is influenced by several risk factors such as immunophenotype (T-ALL, B-ALL), extreme leukocytosis, high leukemia cell proliferation rate, high serum LDH levels, and extramedullary organ involvement. Risk-adapted CNS prophylaxis may be based on these features.

Recently a liposomal preparation of cytarabine has been used for treatment of CNS relapse of lymphoma and ALL and was evaluated in several protocols for prophylaxis of CNS relapse. Due to the preparation a prolonged cytotoxic activity and a more even distribution in the CSF are observed [28]. Therefore fewer applications, i.e., every 2 weeks are required. However combination regimens of liposomal cytarabine with systemic high-dose therapy have to be defined carefully since neurological toxicities may occur [29].

Therapy of Established CNS Disease

About 5–10% of adult ALL patients present with manifestations of CNS leukemia. The incidence is correlated to the immunological subtype and is higher in T-ALL and mature B-ALL. Treatment of overt CNS leukemia is usually undertaken with either i.th. MTX alone, in combination with AC or hydrocortisone, or CRT. I.th. MTX is administered 2–3 times per week and until two consecutive CSF examinations show no evidence of leukemic infiltration. Following the establishment of a remission there is some evidence that continued maintenance i.th. chemotherapy at less frequent intervals is beneficial in prolonging the duration of CNS remission. When adult ALL patients with CNS leukemia at diagnosis are treated adequately, they have no inferior outcome with regard to LFS or CNS relapse rate. However there might be an increased risk of prolonged cytopenias during induction due to the systemic effects of intensified intrathecal therapy.

Stem Cell Transplantation

SCT is an integral part of treatment strategy for adult ALL. Bone marrow and to an increasing extent peripheral blood are used as stem cell source. Despite a great number of trials the indications for SCT in first CR, scheduling, and procedures are still not defined satisfactorily. The potential

advantages of SCT (short treatment duration, favorable outcome in some trials) must be balanced to the disadvantages (mortality of 20–30%, morbidity, late complications, reduced quality of life) and assessed in relation to the improving outcome of conventional and targeted chemotherapy regimens.

The role of SCT in postremission therapy is one major question in the management of adult ALL. The recommended indications for SCT varied over the time [30–32]. With the broader application of pediatric based regimens the indication is defined more restrictively. More recently, the guidelines and many prospective trials are in favor of transplantation in patients with high-risk features. This applies particularly for younger patients treated with intensive pediatric based chemotherapy. In older patients, due to increasing transplant-related mortality, only dose-reduced conditioning regimens can be considered. Future clinical trials will show whether this approach would be superior to chemotherapy regimens.

Overall according to guidelines overall survival for high-risk patients (with varying definitions) was superior for SCT compared to chemotherapy, whereas the role of SCT in standard risk remained open. The need for SCT in specific genetically defined groups of ALL, such as BCR-ABL1-positive or MLL-positive cases, remains to be defined. Allogeneic SCT is currently carried out for MLL-rearranged ALL in most trials and, in the largest study conducted to date, better results have been observed compared with chemotherapy [33]. In younger standard-risk patients, treated with pediatric based regimens, SCT is not recommended by most groups in order to avoid acute mortality and long-term effects.

MRD is considered as the most important factor to guide the decision of chemotherapy or SCT after consolidation. Data from recent studies have shown that SCT offers better results than chemotherapy in patients with high MRD levels after consolidation, regardless of the conventional risk factors at baseline [34]. The question remains open whether SCT is justified in patients with conventional high-risk features but low or negative MRD after consolidation, for whom OS rates ˃50% are expected with chemotherapy. An analysis of the French group demonstrated an advantage of SCT in patients with MRD-positive ALL only [35]. Since historical trials mostly did not consider MRD as a decision tool for SCT, future prospective studies are required to answer this question.

Finally there is a general agreement that SCT is clearly the best therapeutic option for patients in second or later CR [30, 31, 36]. A summary of current indication is given in Table 18.7. Since the outcome of ALL with and without SCT is influenced by numerous factors such as type of SCT, donor match, posttransplant regimens, age, MRD status, and availability of targeted therapies, the indication may change over time.

Table 18.7 Recommendations for SCT in adult ALL (modified from [32, 36])

Treatment phase	Transplant option	Recommendations
CR1	Allogeneic SCT	• Recommended in all patients with persistent MRD • Not recommended in standard-risk patients with sustained molecular remission • Unclear indication in high-risk patients with sustained molecular remission
CR1	Autologous SCT	• Inferior compared to allogeneic SCT and chemotherapy in randomized trials • Maintenance therapy after transplant and negative MRD status may improve outcomes
CR ≥ 2	Allogeneic SCT	• Allogeneic SCT recommended in all patients

Donor Type for Allogeneic SCT

There is sufficient evidence that sibling and very-well-matched, unrelated donors (MUD) SCT can be considered equivalent options in terms of results, and therefore MUD SCT can be offered to patients lacking a sibling donor [32, 36]. Umbilical cord blood can be an alternative source when an HSCT is needed urgently or when the search for a very-well-matched, unrelated donor is unsuccessful [30, 37]. Haploidentical SCT could be an option in patients without a matched sibling or MUD, but prospective comparative studies are lacking. Particularly new approaches for haploidentical SCT with post-transplantation cyclophosphamide provide promising results [38]. Autologous SCT is considered inferior to chemotherapy and to allogeneic SCT [39]. The intensity of pretreatment has an important impact on the outcome of autologous SCT, since it leads to reduction of tumor load. It may still be an option in MRD-negative patients unfit for allogeneic SCT and was successful in Ph-positive ALL [40]. Maintenance therapy after autologous SCT, e.g., with MP and MTX, or imatinib in Ph+ ALL—particularly in MRD-positive patients—is also a useful approach.

Conditioning Regimens

There is no standard MAC regimen, but total body irradiation-based regimens seem to have better antileukemic activity than busulfan-based preparative regimens [30, 41]. Regimens with reduced intensity (RIC) are increasingly considered as an option

for older patients with high-risk ALL or patients with contraindications for myeloablative conditioning [42], but no prospective comparative studies between these two types of preparative regimens have been conducted in young, fit patients.

Factors for Outcome of SCT

Relapse rate and transplant-related mortality both range between 25 and 30%. Although TRM is strongly correlated with age, the upper age limit for SIB–SCT has increased continuously up to 50–55 years. There is evidence that a graft-versus-leukemia (GvL) effect is also present in ALL, as indicated by several observations, such as lower relapse risk in patients with acute and/or chronic GvHD, lower relapse risk after matched unrelated donor SCT, and induction of remission by withdrawal of prophylaxis against GvHD or donor lymphocyte infusions (DLI) in single patients with relapsed ALL.

Furthermore posttransplant monitoring of MRD is of increasing importance to improve the outcome of SCT.

Prognostic Factors in ALL

ALL is not a uniform disease but characterized by subgroups with different biological and clinical features and cure rates. For various parameters prognostic value for either the achievement of remission or for remission duration has been established in adult ALL [1]. At the present time the following are the most important prognostic features: age, initial WBC, immunophenotype, abnormal cytogenetics, or molecular genetics, and to an increasing extent response criteria such as time to achieve CR and MRD (refer to Table 18.10 discussed later in this chapter).

Age

Age is probably the most important prognostic factor. There is a continuous decrease in outcome with increasing age from childhood to elderly ALL patients. In adults, OS ranges up to 70% below 30 years to 20–30% above 50–60 years. In contrast to other prognostic factors age cannot be used to identify patients who could benefit from SCT as done by some groups because outcome of SCT also decreases with increasing age. Actually improved treatment strategies have to be claimed for older patients as well as for young adults with ALL.

Older Patients with ALL

The median survival time in older ALL patients is 3–14 months. There are several reasons that may account for this. Increased hematological and nonhematological toxicity (e.g., hepato- and cardiotoxicity) results in a higher morbidity and mortality during induction therapy. The death rate during induction therapy for older patients above 65 years reaches 20–30%. Incomplete drug administration and extended intervals between cycles of therapy may lead to inferior long-term results. There is a higher frequency of adverse biological features in adults; thus the incidence of Ph+ ALL patients increases from 3% in children to 20–30% in patients above 50 years. The incidence of unfavorable immunophenotypes such as early T-ALL and pro-B-ALL is also higher in older patients. There is, however, no good evidence that within an identical biological subtype of ALL the leukemic blast cells in elderly ALL patients are more resistant than in younger patients.

The optimal treatment for older ALL patients remains to be defined. Registries give an impression on the overall outcome of unselected older ALL patients [43–46]. Survival rates in patients older than 60 years were 10–25% [44, 45] (Table 18.8) with only marginal improvement over the past decades.

30–70% of the older patients are allocated to palliative therapy mainly due to poor performance status at diagnosis. Most studies have shown an advantage of more intensive therapy (Table 18.8). There is no evidence that palliative approaches are associated with lower rates of early mortality or better quality of life.

If older patients are treated according to protocols designed for younger patients, one major problem is the toxicity and early mortality [47]. Patients may acquire severe infections, non-predefined treatment modifications occur frequently, and treatments may be interrupted or even stopped due to severe complications.

More recently several study groups have developed protocols specifically for older ALL patients (Table 18.9) that have the theoretical aim to provide a chance of cure on the one hand and to limit toxicity, early mortality, and hospitalization duration on the other and thereby maintain as much quality of life as possible. Specifically asparaginase seems to be less well tolerated in older patients during induction [48, 49]. Therefore it would be advisable to start asparaginase in older patients later during consolidation.

Table 18.8 Outcome of different strategies to treatment of older ALL patients (adapted from [51])

Approach	Age	*N* studies	*N* patients	CR (%)	Early death (%)	Survival
Population based	≥65	*4*	*n.r.*	40	n.r.	6–30%
Palliative	60–91	4	94	43	24	7 months
Intensive	60–92	12	519	56	23	14%
Age specific	55–85	11	653	72	18	42%

Table 18.9 Prospective specific studies for older ALL patients (adapted from [51])

Author	Year	Age	Patients (N)	CR rate (%)	Early death (%)	OS[a] (%)
Bassan et al. [52]	1996	60–73 (64)	22	59	18	20 (2 years)
Delannoy et al. [53]	1997	55–86 (67)	40	85	n.r.	16 (2 years)
Delannoy et al. [54]	2002	65 (55–81)	58	43	10	n.r.
Offidani et al. [55]	2004	69 (61–79)	17	76	17	38 (2 years)
Sancho et al. [48]	2007	65 (56–77)	33	58	36	39 (1 year)
Kao et al. [56]	2008	66 (60–78)	17	71	29	71 (1 year)
Gökbuget et al. [57]	2008	66 (56–73)	54	85	0	61 (1 year)
Hunault-Berger et al. [58]	2010					
	Arm 1	68 (55–77)	31	90	7	35 (2 years)
	Arm 2	66 (60–80)	29	72	10	24 (2 years)
Gökbuget et al. [50]	2012	57 (55–85)	268	76	14	23 (5 years)
Fathi et al. [49]	2016	58 (51–72)	30	67	3	52 (2 years)
Ribera et al. [59]	2016	66 (56–79)	54	74	14	12; 30 (2 years)[b]

Abbreviations: Ph + Ph/BCR-ABL1-positive ALL included yes or no, Arm 1 continuous infusion doxorubicin, Arm 2 pegylated doxorubicin, *CCR* continuous complete remission, *DFS* disease-free survival, *OS* overall survival
[a]Probability
[b]Estimated from Kaplan–Meier curve

The majority of complications in older ALL patients are observed during induction; thus there is still space for intensification of consolidation therapy. Based on this assumption a consensus treatment protocol for older patients with ALL was defined by the European Working Group for Adult ALL (EWALL) based on a pediatric based protocol developed by the German Multicenter Study Group for Adult ALL (GMALL). The median age of this cohort was 65 years [55–85]. In 268 patients the CR rate was 76%, early death rate 14%, mortality in CR 6%, continuous remission 32%, and survival 23% at 5 years [50]. Patients younger than 75 years with an ECOG performance status below 2 had 86% CR rate, 10% early death, and 36% survival at 3 years.

Overall, pediatric based regimens in ALL are undoubtedly successful and should be scheduled with prospectively defined adaptations with respect to tolerability in older patients. The most important modification of induction therapy in older patients is probably omission of asparaginase, and flexible, reduced dose of anthracyclines. In consolidation, intensified treatment should be attempted and during this treatment phase even asparaginase may be surprisingly well tolerated at moderate doses.

Adolescents and Young Adults with ALL

Several groups have published data comparing the outcome of young adults with "pediatric" protocols to those with so-called adult protocols (most recently in [60]). The reason to select specifically these protocols for comparison is not evident because compared treatment regimens were rather different. The "adult" protocols used for comparison often yielded results below the average; one reason may be that several of these protocols were focused on SCT. In contrast to the studies selected for comparison, several other study groups for adult ALL already apply modified pediatric protocols (Table 18.5) with survival rates of 60% and more in young adults up to 30 years.

These regimens are associated with considerable side effects in adults, particularly liver toxicities and pancreatitis due to asparaginase, polyneuropathies due to vincristine, and avascular bone necrosis. Results are promising at short follow-up but it remains open whether this approach will be feasible, up to which age, at less experienced centers, how selection of patients is handled, and whether it will lead to an improvement of overall results.

On the other hand the attempt of adult ALL study groups to define uniform protocols for ALL ranging from 18 to 75 years is not successful. Currently study groups for adult ALL develop into two directions and [1] either use unmodified pediatric protocols for young adults up to the age of 25–30 years or [2] use modified pediatric protocols, e.g., the BFM-based GMALL protocol for the whole group or adult ALL with additional intensification in young adults. One important aim is dose intensification with a better adherence to time schedules with as few as possible. Another aim is to integrate more successful treatment elements from pediatric trials such as vincristine, dexamethasone, asparaginase, reinduction therapy, and maintenance. The opportunity to use targeted therapies developed in adult ALL studies is useful also for young adults.

White Blood Cell Count

Elevated WBC at diagnosis (>30–50,000/μL) as poor prognostic feature has been confirmed in various trials. The biological reason for the highly resistant behavior of B-precursor ALL with high WBC is unclear. Probably in the future additional molecular markers can help to clarify the underlying mechanisms. Due to the high relapse rate evaluation of MRD, use of experimental drugs and SCT modalities seems particularly important.

Immunophenotype

Immunologic subtypes are associated with different presentation and prognosis and distinct cytogenetic and/or molecular aberrations.

Pro-B-ALL/t(4;11)

Pro-B-ALL and/or t(4;11)-positive ALL is considered as high-risk subgroup in nearly all trials [33]. It appears to be particularly susceptible to high-dose cytarabine-based regimens and SCT as reported from the GMALL studies [61].

Common/Pre-B-ALL

Common(c)/pre-B-ALL bears a large proportion of Ph+ ALL. Based on prognostic factors it can be subdivided into an SR and an HR group with significantly different outcome of 50–60% and 30–40% OS, respectively; 40–50% of patients with common/pre-B-ALL show expression of CD20 on the cell surface. It has been demonstrated that CD20 expression is associated with poorer outcome [62]. Recently promising results have been reported for the addition of rituximab to chemotherapy with a benefit regarding LFS and OS particularly in younger patients [63]. Recently a randomized study confirmed the benefit of rituximab in combination with chemotherapy, which did not impact the CR rates but contributed to higher molecular response rates and improved long-term outcome [64].

T-ALL

Outcome of T-lineage ALL is generally considered superior compared to B-lineage [65]. It comprises the subtypes early T-ALL, thymic (cortical T-ALL), and mature T-ALL with inferior outcome for early T-ALL and favorable outcome for thymic T-ALL. Some groups consider very high WBC (>100.000) also a poor prognostic feature in T-ALL [66].

The biological relevance of immunophenotype is underlined by the fact that overexpression of HOX11, HOX11L2, SIL-TAL1, and CALM-AF10 is associated with subtypes, i.e., maturation states of thymocytes (reviewed in [8]). Some groups observed inferior outcomes for early T-ALL [67, 68] and high expression of the transcription factors ERG and/or BAALC [69]. Low expression of ERG and BAALC was associated with favorable outcome [69] as well as overexpression of HOX11, which is associated with thymic T-ALL [70]. Notch1-activating mutations were identified in up to 50% of T-ALL cases [71, 72]. Notch mutations are correlated with thymic T-ALL and a favorable prognosis. An alternative risk model for T-ALL defines cases with mutations of Notch1 or FBXW7 lacking RAS or PTEN mutations as a favorable subgroup whereas patients with RAS or PTEN mutations lacking Notch1 or FBWW7 mutations are considered as high-risk ALL [73]. The prognostic impact of early T-precursor ALL within early T-ALL remains to be defined [68]. Overall the question of which risk model is most suitable depends on treatment strategies and their historical development in study groups.

Five percent of T-ALL shows the NUP214-ABL1 aberration, which may identify a target population for imatinib therapy [74]. The variety of new prognostic markers can impossibly be integrated in current risk models but may moreover serve to identify pathogenetic mechanisms and therapeutic targets.

With current treatment regimens CR rates of more than 90% and an LFS 50–70% can be achieved in T-ALL. The role of stem cell transplantation within T-ALL remains to be defined [35].

Mature B-ALL

Mature B-ALL is grouped according to the WHO classification together with Burkitt's lymphoma and treated according to a specific concept. Treatment is based on childhood B-cell ALL studies that significantly improved outcome. The drugs responsible for the improvement were high doses of fractionated cyclophosphamide, ifosfamide, HDMTX (0.5–8 g/m^2), and HDAC in conjunction with the conventional drugs for remission induction in ALL, given in short cycles at frequent intervals over a period of 6 months.

The application of these childhood B-cell ALL protocols in original or modified form also brought a substantial improvement for adult patients with B-cell ALL. More than 80% of the cases of mature B-ALL or Burkitt's lymphoma express CD20 on their surface. Further significant improvement of survival rates to 80–90% was achieved by the application of rituximab in combination with chemotherapy [75].

B-cell ALL has a higher incidence of CNS involvement at diagnosis, and of CNS relapse. Therefore, effective measures against CNS disease, such as HDMTX and HDAC as well as intrathecal therapy, are important components of treatment regimens. On the other hand, maintenance treatment has been omitted. Because relapses occur almost exclusively within the first year in childhood as well as in adult B-cell ALL studies, patients thereafter can be considered as cured.

Ph/BCR–ABL-Positive ALL

The translocation t(9;22) and the respective fusion gene BCR–ABL until recently marked the most unfavorable subgroup of adult ALL. Ph/BCR–ABL (Ph+) ALL nearly

exclusively occurs in conjunction with B-precursor ALL (c-ALL, pre-B-ALL). The incidence increases with age. In Ph+ leukemia the BCR–ABL fusion gene is causally involved in leukemogenesis and is considered to be essential for leukemic transformation. With a selective inhibitor of the ABL tyrosine kinase (imatinib) cellular proliferation of BCR–ABL-positive CML and ALL cells can be inhibited selectively. Phase II trial demonstrated a CR rate of 29% in relapsed/refractory Ph+ ALL with imatinib as single drug [76].

The use of imatinib in combination with chemotherapy contributed to a significant improvement in the outcome of newly diagnosed Ph+ ALL. As compared with the pre-imatinib era, CR rates improved from 60–70% to 80–90% or even higher and outcome was much better, with survival reaching approximately 50%, compared with ≤20% in the pre-imatinib era [77–82]. Imatinib in combination with chemotherapy is now considered the standard treatment of Ph-positive ALL.

Historically a subsequent stem cell transplantation was considered as the only chance of cure in patients with Ph+ ALL. It remains a matter of debate whether this is still true in the imatinib era. A recent report confirmed that allogeneic SCT is still associated with a better relapse-free survival in younger Ph+ ALL patients [83]. Younger patients may receive standard myeloablative conditioning, but the role of reduced-intensity conditioning (RIC)-SCT in older patients remains to be prospectively evaluated. After SCT prophylactic imatinib maintenance is probably the best option to prevent post-SCT relapse [84]. The optimal TKI for first-line treatment, and the question whether in patients with favorable response to treatment SCT may be avoided remains matter of discussion.

The incidence of Ph+ ALL increases with age and older patients are usually not candidates for allogeneic SCT. Nowadays, older patients with Ph+ ALL may have a better chance to achieve a CR than patients with Ph-negative ALL. The GMALL study group conducted a first randomized study to evaluate the efficacy of imatinib single-drug induction compared to chemotherapy. The remission rates were 96% and 50%, respectively [85]. The Italian study group focused on first-line therapy with TKI and steroids only. With imatinib (800 mg) and prednisone for induction followed by imatinib single-drug treatment the CR rate, survival, and disease-free survival were 100, 74, and 48% after 1 year [86]. A subsequent trial with dasatinib (140 mg) and prednisone followed by dasatinib single-drug treatment was not specifically designed for older patients (range 24–76 years). The CR rate was 92% and survival was 69%. Post-remission therapy was at the discretion of the treating physician and 14 of 19 patients with TKI monotherapy relapsed with a high frequency of T315I mutations [87].

The largest prospective study so far in older patients with Ph+ ALL used an EWALL chemotherapy backbone with vincristine, dexamethasone, and dasatinib (140 mg) for induction. Consolidation and maintenance according to the EWALL backbone were combined with intermittent dasatinib applications. In 71 patients the CR rate was 96%. The regimen was feasible and the survival after 5 years of follow-up was 36%, which is promising. Persistent MRD above 0.1% after induction and consolidation was associated with a poorer remission duration of only 5 months [88]. A subsequent EWALL trial with a similar backbone but nilotinib (400 mg BID) instead of dasatinib was started subsequently. Again a high CR rate of 97% was reported. Thirty percent of patients achieved a complete molecular remission after induction [89]. Overall, there is increasing evidence that second-generation TKIs in combination with dose-reduced chemotherapy can induce very high CR rates with low mortality in older patients. The rate of molecular remissions appears to be higher compared to imatinib-based regimens. Moderate intensive consolidation therapies in combination with TKIs are tolerated well. Long-term results have to be assessed after 5 or more years and show a still high rate of relapses. New approaches may include reduced intensity SCT, MRD-based change of TKI, or use of new immunotherapies.

Also in younger patients there is a trend towards dose-reduced chemotherapy in induction. A randomized trial showed lower early mortality and higher CR rate in patients receiving imatinib, combined with less intensive chemotherapy compared with those receiving HyperCVAD/imatinib [83]. Once CR has been reached, autologous SCT might also be a good option, at least in patients who have reached a good MRD response, or in those who cannot tolerate allogeneic SCT [83, 90].

In patients with persistent MRD or progressive disease, the recommendation is to switch to another TKI while screening for TKI resistance mutations, and then to adapt TKI choice according to the resistance profile. The third-generation TKI ponatinib is currently the only option in patients progressing with the T315I mutation. First results with ponatinib in newly diagnosed Ph+ ALL are promising but toxicity risks have to be considered, particularly in older, comorbid patients [91].

Treatment Response and Minimal Residual Disease

Longitudinal minimal residual disease (MRD) evaluation during treatment with the aim to assess individual response identifies one of the most important available prognostic factors. The role of MRD as independent prognostic factor has been confirmed in large cohorts of pediatric and

adult ALL (reviewed in [13]). MRD evaluation has two major applications in the management of adult ALL, (1) redefinition of clinical response, failure, and relapse and (2) utilization as prognostic factor, and both are finally used for treatment decisions.

Redefinition of Response

In contemporary trials for adult ALL, CR rates of 85–90% can be reached. The cytological response rate is often favorable, but MRD evaluation reveals differences in subgroups [34]. The molecular CR may be defined as a negative MRD status below 10^{-4} (0.01%) after induction and ranges in adult ALL from 50% for Ph+ ALL treated with imatinib to 80% for SR ALL. Thus molecular CR rate defined according to international standards [14] is an important new endpoint for efficacy evaluation after induction but also after consolidation or salvage cycles (Table 18.6).

During treatment and follow-up molecular relapse, defined as MRD above 10^{-4} after prior achievement of molecular CR, is highly predictive of cytological relapse [34]. Molecular bone marrow relapse is also often present in patients with apparently isolated extramedullary relapse. In clinical trials it should be treated similarly to cytological relapse.

If MRD-based endpoints are used in clinical trials, standardization of methods and definitions is extremely important. It is so far achieved for PCR analysis of individual gene rearrangements [14] and BCR–ABL whereas for flow cytometry or newer methods based on next-generation sequencing international standards for MRD evaluation were not defined so far.

MRD as Prognostic Factor

MRD is a significant prognostic marker at any time point. Early achievement of molecular CR identifies a subgroup of patients with very favorable prognosis. However in the GMALL studies these were only 12% of SR patients [92]. Adult ALL patients reach molecular CR later than children and later time points are more predictive of relapse risk. In the GMALL studies 25–30% of the patients did not achieve molecular CR after induction and first consolidation and nearly all of them relapsed [34]. Similar results were reported by others (reviewed in [13]). SCT can contribute to an improved outcome of patients with high persistent MRD [34, 93]. However patients with high MRD before SCT or persisting MRD after SCT have a poorer outcome [94].

The application of MRD as prognostic factor is complicated by the fact that it has to be combined with "conventional" factors. MRD identifies additional HR patients in those with conventional SR but also good-risk patients in those with conventional HR features, who are usually scheduled for SCT. Thus MRD as prognostic factor depends on time point, treatment protocol, general risk stratification, and planned therapeutic consequences.

Integrated Risk Classification

All risk factors are to a certain extent specific for a defined treatment protocol and used with variations by different study groups. Beyond established factors and factors suggested by individual study groups (Table 18.10), a variety of molecular markers newly detected by microarray analysis have been proposed as prognostic factors [32]. All of these factors can

Table 18.10 Prognostic factors for risk stratification of adult ALL[a]

	Good	Adverse	
		B-lineage	T-lineage
At diagnosis			
Clinical parameters	**WBC < 30,000/μL**	**WBC > 30,000/μL**	WBC > 100,000/μL
Immunophenotype	Thymic T	**Pro B** (CD10−) Pre B (CD10−)	Early T (CD1a−, sCD3−) Mature T (CD1a−, sCD3+)
Cytogenetics/molecular genetics/gene expression profiles	TEL-AML1 HOX11[a] NOTCH-1 9p del Hyperdiploid	**t(9;22)/BCR–ABL** **t(4;11)/ALL1-AF4** t(1;19)/E2A-PBX Complex aberrations Low hypodiploid/near tetraploid	HOX11L2 CALM-AF4 Complex aberrations Low hypodiploid/near tetraploid
Individual response during treatment			
Prednisone response	Good	Poor	
Time to CR	**Early**	**Late (>8–12 weeks)**	
MRD after induction	**Negative/< 10^{-4}**	**Positive > 10^{-4}**	
Age			
	<25 years, <35 years	**>35 years, >55 years, >70 years**	

[a]Generally accepted factors are printed in *bold*

impossibly be integrated in a conventional risk model, which mainly aims to identify patients for SCT in CR1. They may rather stimulate analysis of underlying mechanisms, drug targets, or invention of treatment adaptations. The major aim of future risk stratification in ALL is therefore to identify at diagnosis patients with an increased risk of relapse who are candidates for a stem cell transplantation. The second aim is to identify treatment targets for the use of targeted therapy approaches. During the course of disease the individual response of the patients can be considered by MRD evaluation. This may lead to reconsideration of the indication for SCT, e.g., not to offer a patient with high-risk factors the transplant if he or she is MRD negative or to transfer patients to SCT despite the lack of high-risk factors if he or she remains MRD positive. In the future additional factors such as pharmacogenomic markers or resistance patterns may be considered for treatment stratification.

Treatment of Relapsed or Refractory ALL

Published data show response rates to first salvage of around 40% and overall survival rates below 20% [95, 96]. Patients with late relapse respond to repeated induction therapy in more than 70% of the cases. However, in adult ALL the majority of relapses occur early and the CR rate is clearly below 40%. This was confirmed in a recent international reference analysis [97]. In early relapse the apparent chemotherapy resistance may be overcome by new immunotherapies.

In more than 90% of the cases of B-precursor ALL CD19 is expressed on the surface of blast cells. The bi-specific antibody blinatumomab is directed to CD19 and on the other hand attracts with a CD3 domain cytotoxic T-cells. These cells come in close proximity to the CD19-positive target cells and induce serial killing. A phase II trial in unfavorable cases of relapsed/refractory ALL showed in 189 patients a CR rate of 43% with 82% molecular remissions [98]. The median survival was 6.1 months. In a randomized trial in a similar patient population with early or refractory relapses the CR rate was 44% for blinatumomab compared to 25% for standard chemotherapy. Survival rates were 7.7 months for blinatumomab compared to 4.0 months with standard chemotherapy [99]. More favorable response rates and superior survival were achieved in patients with molecular failure or molecular relapse. The molecular response rate was 78% and the median survival 36 months [100] indicating that the MRD setting is optimal for response and long-term outcome.

Also CD22 is expressed in more than 90% of the cases of B-precursor ALL. The CD22 antibody inotuzumab is conjugated to the cytotoxin calicheamicin. In a randomized trial in relapsed/refractory ALL CR rates of 81% compared to 29% with high-dose cytarabine-based chemotherapy were described. The median survival for patients treated with inotuzumab was 7.7. months [101].

Another promising option is immunotherapy with genetically modified T-cells. These so called chimeric antigen receptor (CAR) T-cells are produced ex vivo from patient-derived T-cells and carry an antigen receptor directed to lymphatic blasts such as CD19 together with several signal transduction elements. The cells are infused after a cytoreductive and immunosuppressive chemotherapy. First results in smaller trials mostly in pediatric ALL cohorts are promising but also demonstrated considerable toxicity [102].

Future treatment of relapsed B-precursor ALL will strongly depend on immunotherapy. However, despite improved response rates, the long-term survival is only around 6 months and the chance of cure strongly depends on subsequent stem cell transplantation. The optimal use of potent new antibody and cell therapies still has to be defined. For individual patients the relapse treatment should be selected based on availability of targeted therapies, age, general condition, type of involvement, and availability of donors. Any longer treatment-free intervals should be avoided. By consequent measurement of MRD and early therapeutic intervention, relapses may be at least partly avoided.

Long-Term Follow-Up and Late Effects

As a result of improved survival rates, more patients with adult ALL are long-term survivors. In pediatric ALL and to a lesser extent adult ALL patients long-term effects of treatment can occur and they should be considered during aftercare of the patients [103]. This includes cataract, infertility, bone necrosis, fatigue, secondary malignancy, hormonal disorder, or psychiatric diseases.

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19 Diagnosis and Treatment of Acute Myeloid Leukemia in Children

Brenton G. Mar and Barbara A. Degar

Introduction

Acute myeloid leukemia (AML) accounts for 15–20% of acute leukemias diagnosed in children and about 5% of childhood cancer diagnoses. Less than 1000 new cases of AML are diagnosed in children each year in the United States [1]. In the past 30 years, survival rates for children with AML have progressively improved, although not as dramatically as for children with acute lymphoblastic leukemia (ALL) [2]. During this period, the components of AML therapy have remained essentially unchanged. Incremental improvements in outcome are attributable to a number of factors including increased treatment intensity, optimized supportive care, and application of stem cell transplantation. With recent advances in molecular diagnostics and the emergence of targeted approaches, AML therapy is poised to enter a new age of accelerated progress. This chapter focuses on factors specifically relevant to AML in children.

Epidemiology and Pathogenesis

AML is primarily a disease of adults, with the median age at diagnosis in the seventh decade of life. In children, the incidence of ALL far exceeds the incidence of AML [1]. Within the pediatric population, the incidence of AML varies by age with a small peak in the first 2 years of life and then gradual rise after the second decade of life, as depicted in Fig. 19.1.

Although the vast majority of pediatric AML is thought to be sporadic in nature, the contribution of inherited leukemia predisposition has become significantly more appreciated over the past decade as more familial myelodysplastic syndrome (MDS), acute leukemia, and marrow failure syndromes have been described. A large genomic study of germline material from 1120 pediatric cancer patients found that 8.5% of all patients and 4% of leukemia patients had a pathogenic or probably pathogenic mutation in a cancer predisposition gene [3]. Importantly, a positive family history of cancer was only present in 40%, demonstrating that germline predisposition remains an underappreciated and under-evaluated concern, which can impact future risk of relapse, second malignancy and other sequelae, transplant conditioning, and transplant donor selection. Although germline screening for predisposition is not (yet) universally recommended for pediatric AML patients, clinicians should have a high index of suspicion for considering a germline workup in the right clinical context. A comprehensive review of these disorders and their diagnostic workup is beyond the scope of this chapter, but we highlight several important syndromes and concepts.

First, there exist cancer predisposition syndromes in which hematological neoplasms are merely one class of several cancers which are at significantly increased risk. These include Bloom syndrome, ataxia telangiectasia, neurofibromatosis type I, and others. In addition, familial MDS and AML syndromes associated with germline mutations have also been described, which can be organized into three groups. In the first group are those with thrombocytopenia, platelet dysfunction, and an increased risk of myeloid and other hematopoietic neoplasms, including constitutional mutations in RUNX1 [4], ANKRD26 [5], and ETV6 [6]. A second group has increased risk of MDS/AML with associated organ manifestations, which include GATA2, TP53, TERT, and TERC. A final group, which includes mutations in CEBPA, SRP72, and DDX41, has increased risk of MDS/AML and no thrombocytopenia and organ dysfunction. Lastly there are bone marrow failure syndromes, including Fanconi anemia, Shwachman-Diamond syndrome ,and dyskeratosis congenita, which also have an elevated risk for transformation to MDS or AML.

Although not familial, Down syndrome deserves special attention as a germline syndrome associated with increased risk of leukemia. Babies with Down syndrome (DS) exhibit

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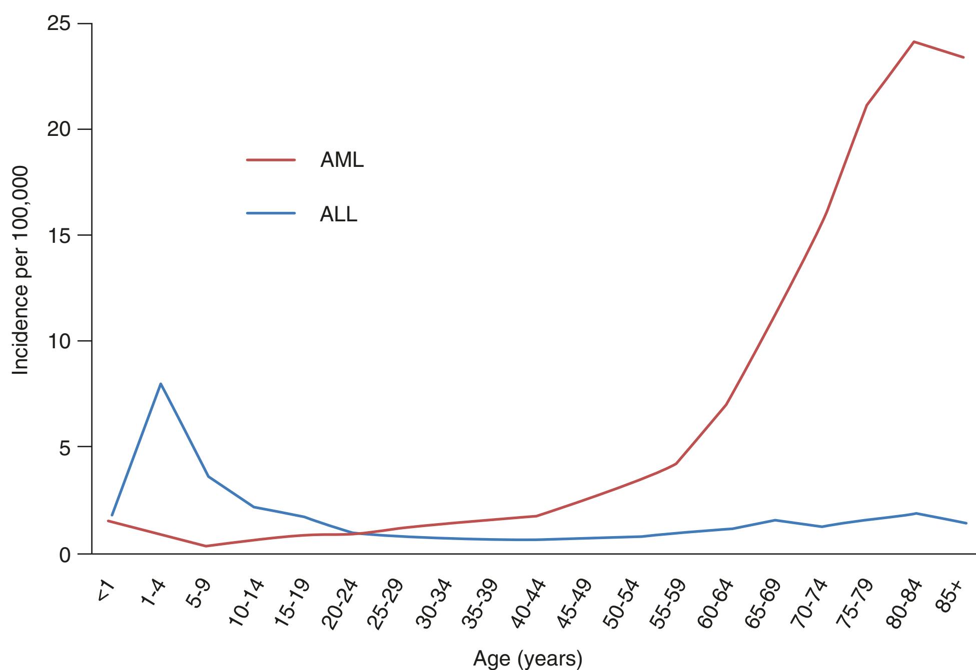

Fig. 19.1 Incidence per 100,000 of acute lymphoblastic leukemia (ALL) and acute myeloid leukemia (AML) by age. Data from Surveillance, Epidemiology, and End Results Program (SEER) 1975–2011

a unique pattern of abnormal myelopoiesis in the neonatal period which is usually transient and self-resolving. Myelodysplastic syndrome (MDS) and/or AML (also known as myeloid leukemia of Down Syndrome, or ML-DS) develops later in some of these babies. After the first few years of life, the incidence of AML declines to non-DS baseline levels concomitant with a rise in the incidence of ALL in children with DS (see ML-DS below).

Increased AML risk is also associated with exposure to DNA-damaging agents in the form of chemotherapy or therapeutic, diagnostic, or environmental radiation. As the number of survivors of childhood and adult cancer grows, the population at risk for this complication increases. The risk period for the development of myelodysplasia and overt AML is influenced by the therapeutic class of the genotoxic therapy previously administered. Also, both cumulative dose and administration schedule appear to matter [7]. Secondary leukemias that arise after exposure to topoisomerase II inhibitors, such as epipodophyllotoxins and anthracyclines, characteristically present as overt AML, without a preceding phase of myelodysplasia. These leukemias occur relatively early, peaking at 2–3 years after drug exposure. Balanced translocations, especially rearrangements involving the MLL (KMT2A) gene at chromosome 11q23, are characteristic of AMLs that occur in association with these agents [8]. Frequent, intermittent schedule of administration of etoposide is associated with a higher risk of treatment-related leukemia [9]. In contrast, exposure to alkylating agents is characteristically associated with leukemias that evolve in the setting of MDS occurring more than 5 years after drug exposure. Deletions involving chromosomes 5 and 7 are often observed [8].

Other environmental and lifestyle factors do not appear to contribute strongly to the incidence of AML in children. Studies linking childhood AML to exposure to environmental toxins, pesticides, fetal exposure to cigarettes, drugs or alcohol, parental age, and birth weight have been suggestive but so far inconclusive [10].

Clinical Presentation

Children with acute leukemia, whether AML or ALL, typically present with symptoms related to bone marrow infiltration, including pallor, fatigue, fever, petechiae, and bruising. Hepatosplenomegaly is common. Less often, AML cells can also form solid masses, referred to as myeloid sarcoma or chloroma, or infiltrate tissues such as the gingiva and skin. This may occur with or without concomitant peripheral blood or bone marrow involvement. Extramedullary involvement is associated in particular with young age, monoblastic differentiation, and certain cytogenetic abnormalities [11].

In children with AML, the white blood cell count at presentation may be low, normal, or high and circulating blasts may be present or absent. 15–20% of children present with a white blood cell count of 100,000 cells/μL or more [12–14]. Rarely, circulating blasts are detected on screening blood work performed in the absence of symptoms. When the white blood cell count is extremely elevated, patients may experience signs and symptoms related to impaired tissue perfusion that results from hyperviscosity and microvascular obstruction. Leukostasis is a significant risk in AML patients with white blood cell counts over 100,000. Respiratory

compromise and central nervous system (CNS) manifestations are of primary concern. Concomitant renal dysfunction and metabolic derangement due to spontaneous tumor lysis syndrome and hemorrhagic diathesis due to thrombocytopenia and disseminated intravascular coagulopathy (DIC) exacerbate the problem. As in adults, DIC with or without hyperleukocytosis is a particularly prominent feature of acute promyelocytic leukemia (APL). The role of leukapheresis remains controversial in children with hyperleukocytosis. In conjunction with prompt initiation of cytoreductive chemotherapy and aggressive management of tumor lysis syndrome, the procedure might reduce the risk of early death in selected patients with very high WBC (>200,000) and monoblastic leukemia [15]. Due to risk of bleeding, leukapheresis is discouraged in patients with APL [16].

The diagnosis of AML may be established when leukemic blasts are circulating in the peripheral blood. However, bone marrow aspiration/biopsy is usually recommended to fully characterize the leukemia. In every new case of AML, the possibility of APL must be considered, since this entity demands urgent initiation of specific therapy (see below). Leukemia cytogenetics, FISH, and molecular tests for subchromosomal genetic alterations, such as FLT3-ITD, should be obtained. Although institutional protocols may differ on which exact studies are sent, molecular studies are increasingly important in determining treatment and predicting prognosis. The option to bank leukemia samples for future studies should always be offered when possible.

Examination of CSF is an important part of the diagnostic evaluation in new-onset leukemia, although it may be delayed or deferred in the setting of coagulopathy. CSF sampling is usually performed in conjunction with administration of intrathecal chemotherapy when the diagnosis of acute leukemia has been established. A summary of commonly recommended studies for the diagnostic evaluation of pediatric AML is shown in Table 19.1.

Table 19.1 Diagnostic evaluation of suspected pediatric AML

Initial workup and staging
• History and physical exam, including family history for malignancies and hematological disorders
• Complete blood count with differential
• Electrolytes, including calcium, phosphorous, uric acid, and lactate dehydrogenase
• Liver function tests, including AST, ALT, bilirubin
• Prothrombin time (PT), partial thromboplastin time (PTT), international normalized ratio (INR), fibrinogen, d-dimer
• Peripheral blood flow cytometry, if circulating blasts are present
• HLA typing for potential transplant
• Chest radiograph
• Ophthalmology exam
• Echocardiogram, electrocardiogram
• Consider central access options
• Consider fertility-preservation options
• Consider research specimen banking, if available
• Bone marrow aspirate
– Morphology, flow cytometry, karyotype, FISH for prognostic AML cytogenetics including t(8;21), inv. [16], MLL, t(15;17), −7, +8
– FLT3 internal tandem duplication
– PML/RAR if suspected or confirmed APL (consider also from blood)
• Bone marrow biopsy
• If no coagulopathy, diagnostic/therapeutic lumbar puncture

Classification

AML is a morphologically, cytogenetically, and molecularly heterogeneous set of diseases and its classification strongly influences prognosis and therapy. Many classification schema have been developed over time, for example, the French-American-British (FAB) classification system, which used morphologic criteria to divide AML into eight groups (M0: acute myeloblastic leukemia with minimal differentiation, M1: acute myeloblastic leukemia without maturation, M2: acute myeloblastic leukemia with maturation, M3: acute promyelocytic leukemia, M4: acute myelomonocytic leukemia, M5: acute monocytic leukemia, M6: acute erythroid leukemia, and M7; acute megakaryocytic leukemia) [17, 18]. However, with increasing understanding of AML biology, particularly in adults, recurrent genetic and cytogenetic alterations have become integrated with classification. This is reflected in the introduction of World Health Organization (WHO) classification of myeloid neoplasms and acute leukemia in 2001, which was revised in 2008 and again in 2016 (Table 19.2).

Because AML is primarily a disease of adults, it is not surprising that the WHO classification schema has limited applicability to pediatric AML. This is illustrated by the fact that in a study of 639 children with AML, AML-NOS (not otherwise specified) is the largest group [19].

Cytogenetic and Molecular Features of Childhood AML and Their Role in Risk Stratification

In large part, the limited applicability in pediatric AML of the genetic and cytogenetic approach driving the WHO classification is driven by age-related differences in the molecular features of adult and childhood AML. Many of the common cytogenetic abnormalities vary considerably by age (Fig. 19.2), and common pediatric alterations that are uncommon in adult AML are not well represented in the WHO classification. For example, although *KMT2A (MLL)* has over 120 described fusion partners [6, 7] and is translocated in

Table 19.2 World Health Organization 2016 classification of myeloid neoplasms and acute leukemia

Acute myeloid leukemia (AML) and related neoplasms
AML with recurrent genetic abnormalities
AML with t(8;21)(q22;q22.1);RUNX1-RUNX1T1
AML with inv. [16](p13.1q22) or t(16;16)(p13.1;q22);CBFB-MYH11
APL with PML-RARA
AML with t(9;11)(p21.3;q23.3);MLLT3-KMT2A
AML with t(6;9)(p23;q34.1);DEK-NUP214
AML with inv. [3](q21.3q26.2) or t(3;3)(q21.3;q26.2); GATA2, MECOM
AML (megakaryoblastic) with t(1;22)(p13.3;q13.3);RBM15-MKL1
Provisional entity: AML with BCR-ABL1
AML with mutated NPM1
AML with biallelic mutations of CEBPA
Provisional entity: AML with mutated RUNX1
AML with myelodysplasia-related changes
Therapy-related myeloid neoplasms
AML, NOS
AML with minimal differentiation
AML without maturation
AML with maturation
Acute myelomonocytic leukemia
Acute monoblastic/monocytic leukemia
Pure erythroid leukemia
Acute megakaryoblastic leukemia
Acute basophilic leukemia
Acute panmyelosis with myelofibrosis
Myeloid sarcoma
Myeloid proliferations related to Down syndrome
Transient abnormal myelopoiesis (TAM)
Myeloid leukemia associated with Down syndrome
Acute leukemias of ambiguous lineage
Acute undifferentiated leukemia
Mixed-phenotype acute leukemia (MPAL) with t(9;22)(q34.1;q11.2); BCR-ABL1
MPAL with t(v;11q23.3); KMT2A rearranged
MPAL, B/myeloid, NOS
MPAL, T/myeloid, NOS

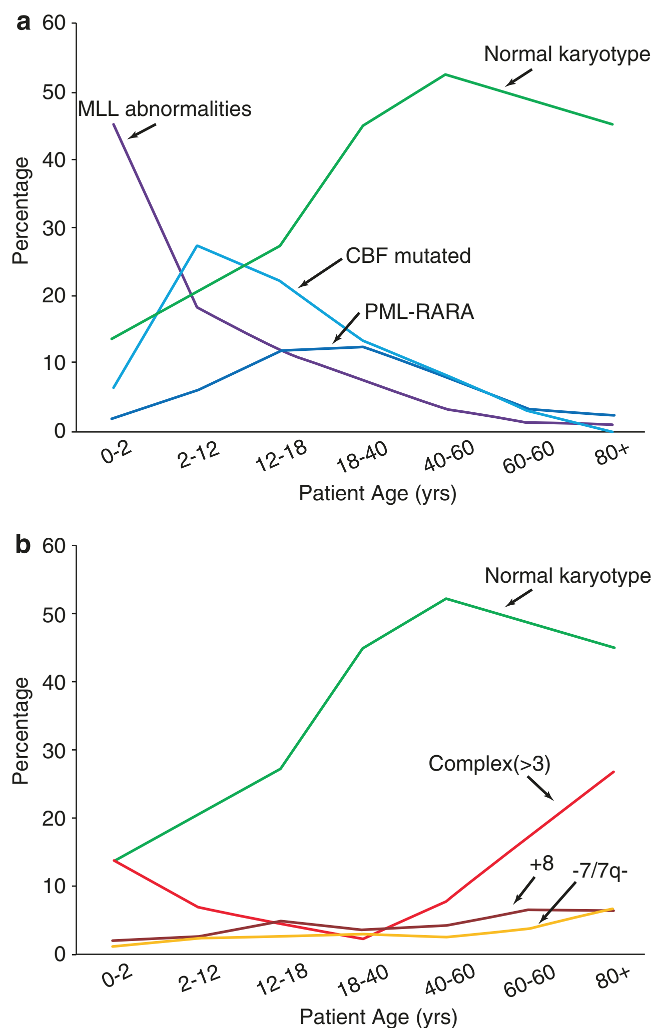

Fig. 19.2 Frequency of molecular abnormalities in AML by age. (**a**) Abnormalities more common in pediatric AML, with normal karyotype AML as a reference. (**b**) Abnormalities more common in adult AML. Data summarized from Cruetzig et al. Cancer 2016

50% of infants and 25% of children with AML, it is uncommon in older adults. As a consequence, the WHO criteria in 2008 and 2016 recognize only the most common fusion protein, t(9;11)(p21.3;q23.3); MLLT3-KMT2A (previously known as MLL-AF9) [8], leading AMLs with other KMT2A translocations to be classified as AML-NOS, despite their differential impact on prognosis.

Other pediatric centric molecular features are recognized however, for example, the favorable risk core-binding factor (CBF) group, with t(8;21) or inv.(16) and t(16;16), is very common (>25%) in childhood AML, while uncommon in older adults with AML. PML-RAR is common (>10%) in adolescents and young adults with AML, while uncommon in young children and older adults over 60. In contrast, cytogenetically normal, complex karyotype and losses of 5q and 7 are more common in older adults.

Although the frequencies of specific cytogenetic abnormalities vary, most abnormalities retain a similar clinical meaning in pediatric compared to adult AML (Table 19.3). However there are a few notable exceptions. Both monosomy 7 and 7q- are poor risk markers in adults, while children with 7q- do relatively well compared to those with monosomy 7 [19–21]. Since 11q23 rearrangement is more common in children, more data is available regarding the prognostic impact of specific KMT2A translocations in pediatric AML, which is variable. The t(6;11)(q27;q23), t(10;11)(p12;q23), and t(10;11)(p11.2;q23) translocations have been specifically associated with unfavorable outcomes, while

Table 19.3 Prognostic cytogenetic alterations in pediatric acute myeloid leukemia

Favorable	
t(8;21)(q22;q22)/RUNX1-RUNX1T1	[21, 24]
Inv(16)(p13.1;q22)/CBFb-MYH11	[21, 24]
Mutated NPM1 without FLT3-ITD	[25, 26]
Biallelic mutations of CEBPA	[27, 28]
t(1;11)(q21;q23)/MLLT11-KMT2A	[22]
t(15;17)/PML-RARA	[24]
Intermediate or unclear	
t(9;11)(p12;q23)/MLLT3-KMT2A	[22]
Other KMT2A fusions	[22]
Unfavorable	
t(6;11)(q27;q23)/MLLT4-KMT2A	22
t(10;11)(p12;q23)/MLLT10-KMT2A	[22]
t(10;11)(p11.2;q23)/ABI1-KMT2A	[22]
t(6;9)(p23;q34)/DEK-NUP214	[29, 30]
t(5;11)(q35;p15.5)/NUP98-NSD1 with FLT3-ITD	[31, 32]
Inv(16)(p13.3q24.3)/CBFA2T3-GLIS2	[33, 34]
FLT3-ITD	[26, 35]
Monosomy 7	[19, 20, 21]
t(9;22)/BCR-ABL	[21]
5q abnormalities	[21]

t(1;11)(q21;q23) is favorable, and others appear to have little impact on prognosis [22]. The prognostic significance of other cytogenetic lesions is emerging in pediatric AML, but is not yet validated [23].

In addition to cytogenetic alterations, nearly all cases of adult AML have multiple pathogenic somatic single-nucleotide variants (mutations) and some copy number variants that can be identified by next-generation sequencing techniques, with the most frequently altered including *FLT3, NPM1, DNMT3A, IDH1, IDH2, TET2, RUNX1, TP53, CEBPA, WT1, KIT*, the Ras pathway (*NRAS, KRAS, PTPN11*), splicing (*U2AF1, SF3B1, ZRSF2*), and the cohesion complex (*STAG2, SMC3, RAD21*) [36]. Although studies have been limited to small cohorts, often assaying only a few genes, variants in most of these genes have been far less common. For example, *FLT3-ITD* (25% vs. 12%) and mutations in *CEBPA* (35% vs. 8%), *IDH1* or *IDH2* (17% vs. 2%), and *DNMTA* (22% vs. 0%) [37] are all significantly more frequent in adult versus childhood AML patients.

The cell of origin, order, number, and which combinations of mutations are required for AML development remain an active field of investigation, beyond the scope of this chapter. Gilliland and colleagues previously proposed a model in which AML develops from a combination of "class II" mutations, which primarily cause a differentiation arrest and increased self-renewal, with "class I" mutations that cause increased proliferation and survival [38]. Such class I mutations include activating mutations in the Ras pathway as well as constitutive activation of receptor or cytoplasmic tyrosine kinases such as *FLT3* and *KIT*. The contribution of many recently described mutations, such as those in epigenetic regulators, splicing, and cohesin complex, are incompletely understood and their effects on differentiation, self-renewal, and proliferation are under investigation.

Increasing evidence is accumulating that some of these somatic variants, particularly in *DNMT3A*, *TET2*, *ASXL1*, *JAK2*, and *SF3B1*, may be an initial event in the pathogenesis of adult AML. Several studies [39–41] have now documented the existence of detectable, pathogenic, persistent, and typically subclonal variants in these genes in the blood of healthy volunteers, which likely represent the expansion of mutated hematopoietic stem cells and is now termed clonal hematopoiesis of indeterminate potential (CHIP) [42, 43]. These mutations are associated with an increased risk of transformation to a hematopoietic malignancy; they are more frequent in patients with unexplained cytopenias [44] and have been documented in non-leukemic hematopoietic stem cells of leukemic patients [45] and the blood samples of patients prior to the development of AML [46]. The prevalence of CHIP increases dramatically with age, with over 10% affected of those over 70 years or older, but is rare in healthy adults under 40 [39], which is thought to be related to age-related accumulation of DNA damage. The near absence of these variants in healthy young people and low prevalence in childhood AML suggest that most childhood AML may have a very different etiology than most adult AML.

Immunophenotype Analysis at Diagnosis in Pediatric AML

The diagnosis of AML, to be distinguished from ALL and MPAL, has been greatly assisted by the advent of flow cytometry and immunophenotyping. An aberrant immunophenotype can be detected on myeloid blasts in 95% of cases [47] at diagnosis, commonly with expression of myeloid and stem cell markers CD13, CD15, CD33, CD34, CD56, and CD117 as well as lymphoid markers such as CD7, CD10, and CD19. Some specific immunophenotypes are correlated to cytogenetics or outcomes and should be noted.

Minimally differentiated M0 FAB subtype of AML is rare and a poor prognostic marker [48]. The good prognostic t(8;21)(q22;q22) translocation is more frequently found in pediatric patients, in the FAB M2 subtype, and often associated with extramedullary tumors, chloromas, and splenomegaly. They also have a specific immunophenotype, with positivity for the B-cell cell-associated marker CD19, as well as CD13, CD34, CD56, and HLA-DR, while CD2 and CD7 are rarely expressed and CD33 is weak [49].

In FAB M3, acute promyelocytic leukemia (APL) blasts co-express CD13, CD33, and CD9 but are negative for HLA-DR, CD15, CD10, or CD11b. In addition to distinct morphologic features, this immunophenotype will be one of the earliest hints that a newly presenting patient has APL and could benefit from the early initiation of tretinoin (see below).

M6 erythroleukemia is rare in childhood AML but associated with a poor outcome, particularly induction failure and death [50]. The megakaryocytic M7 FAB subtype may be suspected based on morphology or histochemistry, and is confirmed by immunophenotypic analysis (identification of platelet or megakaryocytic antigens CD41 and CD61) [18]. It is frequently associated with Down syndrome, and is in fact the most common type of AML in young children with Down syndrome, where it has a favorable prognosis. In contrast, M7 AML in non-Down syndrome patients is rare and carries a poor prognosis [50].

Loken and colleagues [51] recently described a pediatric restricted high-risk immunophenotype with bright CD56, dim to negative CD45 and CD38, and a lack of HLA-DR, named the RAM phenotype (after a particular patient's initials). These patients were significantly younger (1.26 years old vs. 10.1) and lacked FLT3-ITD, CEBPA, or NPM1 mutations and were all considered standard-risk cytogenetics; however, they were more frequently MRD positive (84% vs. 33%), had lower EFS (16% vs. 51%), and OS (26% vs. 66%) compared to the non-RAM cohort.

Treatment

Treatment of AML in children is similar to adults, but outcomes in children are superior in all risk groups [12, 52]. Overall survival for pediatric AML exceeds 60% with current treatment protocols [23]. Treatment is separated into two phases: remission induction and post-remission consolidation. Approximately 90% of children achieve remission after one or two cycles of intensive induction chemotherapy [53]. The choice of post-remission consolidation is determined by risk group which is, in turn, based on the combination of disease characteristics and treatment response. Broadly speaking, lower risk patients in first complete remission are allocated to receive additional cycles of combination chemotherapy while higher risk patients are typically referred for allogeneic hematopoietic cell transplantation (HCT). Allocation to HCT is not based solely on the existence of a matched family donor, as it was in the past. For AML patients who relapse, HCT in second remission is generally considered the best option for the possibility of cure.

Remission Induction

For decades, the combination of cytarabine plus an anthracycline has remained the mainstay of induction therapy for newly diagnosed AML. Multiple clinical trials in childhood AML have examined different cytarabine doses and schedules, different anthracyclines, and addition of a third agent. Despite these efforts, no clear optimal combination and schedule have been defined. Currently in the United States, induction therapy typically consists of cytarabine, daunorubicin, and etoposide (ADE 10 + 3 + 5) based on the series of trials conducted by the United Kingdom Medical Research Council (MRC) [54, 55]. Bone marrow examination is performed to assess response to chemotherapy around days 21–28 of induction I. If residual leukemia is present, a second cycle of chemotherapy may be immediately initiated. Otherwise, induction II begins when peripheral blood counts recover. Morphologic assessment of early treatment response is notoriously challenging. Application of multiparameter flow cytometry and molecular studies significantly improves the accuracy of the hematopathologists' interpretation [56]. Depending on genetic and clinical factors, response to induction I, and provider preference, a second cycle of ADE or a more intensive combination, such as high-dose cytarabine with mitoxantrone [13], is administered in induction II. After two cycles of induction chemotherapy, about 90% of children with de novo AML will achieve remission, defined as <5% marrow blasts by morphology and/or flow cytometry. Among the subset of patients who do not achieve this milestone, refractory disease is the cause in the majority, in most studies. However, early death due to toxicity is also a significant problem [14, 57, 58].

Monitoring Treatment Response

In addition to the initial immunophenotypic, cytogenetic, and genetic features, response to therapy is a strong predictor for relapse and long-term outcome. Morphologic induction failure, unquestionably, portends a dismal prognosis, but occurs in only a small minority of cases and unfortunately nearly half of patients that achieve complete remission (CR) will eventually relapse. Modern assessment of treatment response involves the measurement of minimal residual disease (MRD), which is typically performed by sensitive multidimensional flow cytometry after induction chemotherapy; however RT-PCR analysis of leukemia-specific transcripts [59] and genomic DNA assessment of mutation clearance have been studied as well.

Flow cytometry for aberrant immunophenotypes post-induction is the most generalizable method and has been shown to be prognostic in childhood AML and predicts relapse risk in multiple studies. For example, in the St. Jude AML02 trial [58], the presence of high MRD by flow after one course of induction was associated with a 3-year cumulative incidence of relapse of 39% compared with only 17% for patients with no detectable MRD. The relapse rate was particularly high for patients with MRD >1% after one course of therapy and for those with any detectable MRD (>0.1%) after two courses of induction chemotherapy.

At relapse, 88% of pediatric AML cases demonstrated an antigenic shift in at least one marker [60]. Because immunophenotype can vary greatly and aberrancy can be subtle, it is important to perform multiparameter flow cytometry, especially in cases of minimal disease detection, which should not be restricted to the immunophenotype detected at presentation. In contrast to adult AML trials, flow-based MRD is well accepted and used by most pediatric cooperative group to determine which patients receive high-risk therapy (in combination with clinical, cytogenetic, and genetic features).

Consolidation Chemotherapy

Consolidation therapy for pediatric AML in first complete remission is determined by risk group. It is generally agreed that the core-binding factor (CBF) leukemias have a reasonably good chance of cure with chemotherapy alone. For these patients with so-called low-risk cytogenetics, the up-front toxicity of HCT in first complete remission is not warranted [61, 62]. On the opposite end of the spectrum, high-risk AML is variably defined among cooperative groups and the definition continues to evolve. General consensus has emerged for many of the more frequent subtypes and scenarios, taking into account both disease features and response to therapy. High-risk characteristics include poor response to initial therapy, AML arising in the setting of MDS or prior treatment, presence of FLT3-ITD with high allelic ratio, and presence of adverse cytogenetics such as monosomy 7, monosomy 5, del5q, and other specific rare translocations [14, 55, 63–65]. HCT from the best available donor is typically, although not universally, considered the treatment of choice for children with high-risk AML in first complete remission, even though a clear benefit from HCT has been difficult to demonstrate in prospective [66] and retrospective trials [67]. A loosely defined intermediate-risk group is comprised of patients with neither low-risk nor high-risk features. For this population, the risk-benefit analysis related to consolidation chemotherapy versus HCT is closer to neutral. Individual factors, such as the availability of a matched family donor, may be taken into consideration in selecting the best consolidation strategy.

For patients who do not proceed to HCT in first remission, post-remission treatment usually consists of additional cycles of intensive consolidation chemotherapy, including some exposure to cytarabine at high dose (≥1 g/m^2/dose) [65, 68]. Although the optimal number of cycles is not determined, a total of four cycles of multiagent chemotherapy is relatively standard in the United States at this time. This is primarily based on results from the MRC AML12 trial which demonstrated that three cycles of consolidation chemotherapy did not improve event-free or overall survival in comparison to two cycles [55]. With current intensive chemotherapy, children with CBF leukemia can expect event-free survival in the range of 80% and overall survival in the range of 90% at 3 years [13, 58]. The influence of risk group on outcome is depicted in Fig. 19.3 [58]. Maintenance chemotherapy has no proven benefit in childhood AML [69, 70], with the exception of APL [71].

Extramedullary Disease

The incidence of central nervous system (CNS) involvement is higher in children with AML compared to ALL. However, the impact that it has on prognosis, and therefore on treatment, is less important. CNS involvement in AML is associated with higher presenting WBC count, younger age, and certain karyotypic abnormalities. CNS-directed therapy in the form of intrathecal chemotherapy, in addition to CNS-penetrant systemic chemotherapy is incorporated into all contemporary treatment protocols for pediatric AML. Prophylactic cranial radiation is not routinely used. CNS3 status, which is defined as ≥5 WBC/μL and blasts present on cytospin, occurs in about 10% of children with AML [72]. For these children, intensification of intrathecal chemotherapy is recommended. The management of CNS2 status (<5 WBC with blasts seen) is variable. Although CNS involvement does not appear to confer a significant impact on overall prognosis, it is associated with a higher risk of CNS relapse [72, 73].

Soft-tissue masses, referred to as myeloid sarcoma or chloroma, are identified occasionally in children with newly diagnosed AML. The pattern and prognostic impact of this are linked to associated karyotypic changes. As mentioned above, cutaneous involvement (“leukemia cutis”) is characteristically seen in babies with *KMT2A (MLL)*-rearranged AML (Fig. 19.4). Orbital, parameningeal, and CNS masses

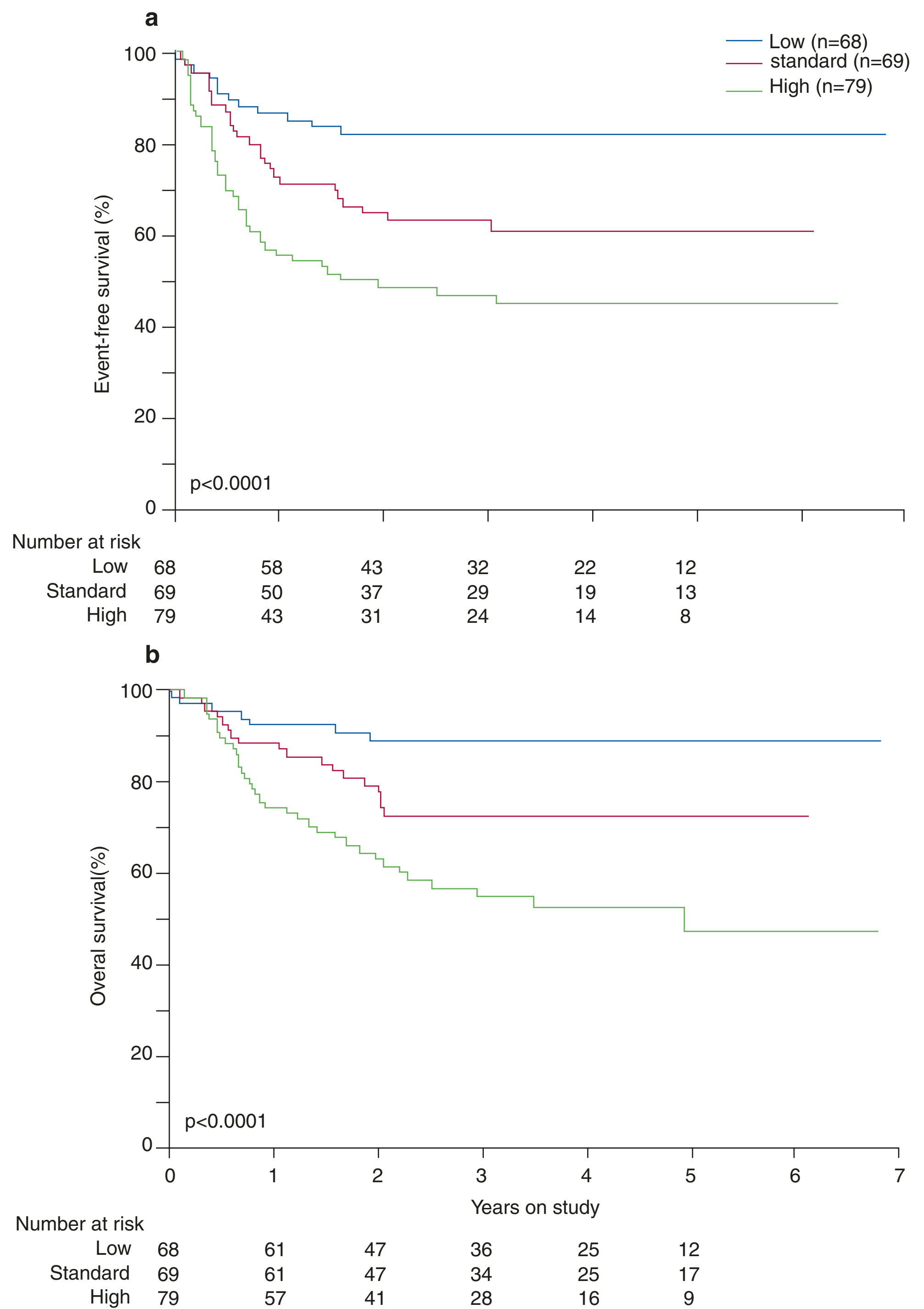

Fig. 19.3 Treatment outcome according to risk group on St. Jude AML02. Reproduced with permission from Rubnitz, et al. Lancet Oncology 2010

are characteristic of AML in older children in association with favorable cytogenetics [74]. Although focal radiation may have a role in selected cases for the acute management of myeloid tumors that threaten permanent consequences due to their location or in the palliative setting, there is no established benefit to addition of focal radiation to myeloid tumors that respond well to chemotherapy [75]. Apparently "isolated" extramedullary disease is viewed as a harbinger of systemic illness both at initial diagnosis and at relapse and should be treated as such [76].

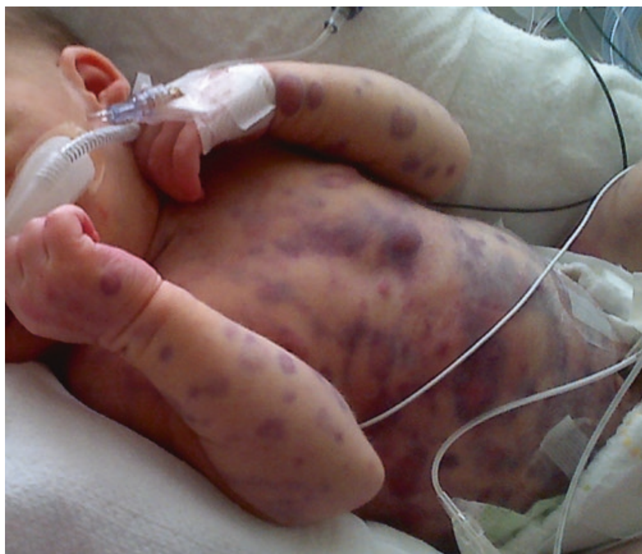

Fig. 19.4 Leukemia cutis in a newborn infant with KMT2A (MLL)-rearranged AML

Newer and Targeted Agents

Due to the suboptimal outcomes in pediatric AML, novel therapies targeting a broad variety of mechanisms are in development. These include second-generation nucleoside analogs, such as clofarabine, which is more resistant to deamination than classical agents such as cytarabine [77], new formulations of traditional chemotherapy, such as CPX-351, a liposomal formulation of daunorubicin and cytarabine, and targeted agents such as kinase inhibitors, monoclonal antibodies, epigenetic modulators, and others. Since AML is much more common in adults, novel therapies are generally studied and often shown to have activity in adults before being used in pediatric studies.

As noted above, the presence of the FLT3 internal tandem duplication is an adverse prognostic feature in children [35, 78] as it is in adults. Several FLT3 inhibitors that range in specificity and potency already exist that have been, or are being, tested in adult AML. These drugs include midostaurin, sorafenib, quizartinib, gilteritinib, and crenolanib [79]. The multikinase inhibitor midostaurin was approved for adults with AML with certain FLT3 mutations in 2017, however data in children is limited at this time. The multikinase inhibitor sorafinab, which is FDA approved for certain solid tumors, also has activity against some FLT3 mutations, with reported clinical activity in relapsed pediatric AML [80] and some use in the post transplant setting [81].

Gemtuzumab ozogamicin is a humanized monoclonal antibody directed against CD33, a protein that is expressed on the surface of a high proportion of AML blasts but not on hematopoietic stem cells. The antibody is covalently linked to an antitumor antibiotic, calicheamicin. The drug was approved in 2017 for AML in patients aged 2 and older [82]. Children's Oncology Group (COG) AAML 0531 [14] demonstrated that addition of gemtuzumab was associated with a statistically significant positive effect on event-free survival through reduction of relapse. High expression of the target antigen, CD33, is associated with response to the drug [83]. Additional immunoconjugates, including vadastuximab talirine (SGN-CD33A) [84], are in development for AML.

Many other classes of agents show promise in AML and are currently under investigation. Epigenetic modifiers are a broad class including drugs that inhibit histone deacetylase (vorinostat) and DNA methyltransferase (azacitidine and decitabine) which are being studied in relapsed pediatric and adult AML. DOT1L inhibition is being studied in children and adults for KMT2A (MLL)-rearranged AML. Other strategies being investigated with new clinical agents include IDH1 and IDH2 inhibition, proteasomal inhibition, exportin inhibition, ubiquitination modulation, and chimeric antigen receptor T-cell therapy [53, 85].

Acute Promyelocytic Leukemia

Acute promyelocytic leukemia (APL) is a unique AML subtype characterized by the presence of reciprocal translocation involving chromosomes 15 and 17 leading to the production of a promyelocytic leukemia (*PML*)-retinoic acid receptor alpha (*RARA*) fusion protein. APL is a disease of older children and young adults. APL accounts for about 10% of pediatric AML and it is distinctly uncommon in children under 10 years. The molecular features of APL are described above and elsewhere in this text. In children as in adults, the treatment of APL differs significantly from other AML subtypes and the overall prognosis is markedly better. But APL is associated with a high risk of early death, due primarily to hemorrhage. Therefore, clinicians must be on alert for this entity because prompt and accurate diagnosis is critical to successful management.

APL is characterized morphologically by the appearance of large hypergranular myeloid blasts with prominent Auer rods. The hypogranular variant (M3v), which is more common in children [86], may be more difficult to distinguish from other AML subtypes. Typically, the blasts co-express CD13, CD33, and CD9 but do not express HLA-DR, CD15, CD10, or CD11b. Blasts of the hypogranular variant typically express CD2 [87]. Demonstration of the molecular fusion of PML gene on chromosome 17 and the RARA gene

on chromosome 15 by RT-PCR, FISH, or karyotype is the key to diagnosis. RT-PCR is the preferred modality since it is rapid, specific, and quantitative.

Disseminated intravascular coagulopathy (DIC) is a significant and potentially life-threatening feature of new-onset APL. Transfusions should be provided to maintain the platelet count ≥30–50 K and fibrinogen level ≥100–150 mg/dL. To address the underlying cause of the bleeding diathesis, emergent initiation of leukemia-directed therapy in the form of tretinoin is recommended at the first suspicion of the disease [16]. Delay in starting tretinoin is associated with a higher risk of early hemorrhagic death [88]. In children, there is an estimated 7.4% risk of death within the first 7 days of APL diagnosis [89].

The introduction of tretinoin into the treatment of APL in the late 1980s transformed the management and the prognosis of this disease. Tretinoin is now a standard component of APL therapy in children. Tretinoin causes differentiation of malignant promyelocytes, sometimes leading to marked increase in the peripheral leukocyte count. Differentiation syndrome occurs in 10–20% of children being treated with tretinoin for APL [90–92]. Higher presenting WBC is a risk factor for the development of this complication of therapy which is characterized by fever, hypotension, pulmonary infiltrates, and renal insufficiency. Aggressive supportive care, administration of dexamethasone, and early introduction of cytotoxic chemotherapy are recommended. Temporary interruption of tretinoin is indicated in severe cases. Pseudotumor cerebri is an important side effect of tretinoin that occurs more commonly in children than in adults [90–92] warranting the use of a lower starting dose of tretinoin in children.

While it is true that APL is associated with an unacceptably high risk of early morbidity and mortality, the overall prognosis for children with APL is significantly better than most other AML subtypes. Treatment with combination of chemotherapy and tretinoin results in event-free and overall survival in the range of 75 and 90%, respectively [90, 91]. As in adults, the intensity of treatment is risk stratified based on presenting white blood cell (WBC) count, with high risk defined as presenting WBC >10,000 cells/dL. Although highly effective, these chemotherapy regimens are lengthy (almost 3 years in duration due to inclusion of a maintenance phase) and associated with potential for significant late effects (especially cardiotoxicity related to high cumulative anthracycline exposure). Exciting recent studies in adults have demonstrated that APL can be cured in many cases with combination of tretinoin and arsenic trioxide with little or no cytotoxic chemotherapy [93]. Arsenic has been shown to be effective and tolerable in children with relapsed APL [94] and in small series of children with newly diagnosed APL [95, 96]. Ongoing clinical trials are examining the use of arsenic and tretinoin as up-front therapy for pediatric APL with concomitant reduction or omission of cytotoxic agents and shortening of treatment duration.

Myeloid Leukemia of Down Syndrome

Children with Down syndrome (DS) are at a 10–20-fold increased risk for developing leukemia. The pattern of disease is unique, comprising neonatal transient abnormal myelopoiesis (TAM), MDS/AML, and ALL. In the first 5 years of life, the risk of myeloid leukemia is about 150 times higher in DS children compared with non-DS children [97].

Myeloid blasts are identified in the peripheral blood smears of approximately 10–15% of newborns with DS. The blasts typically, but not always, express immunophenotypic markers of megakaryoblasts, including CD41, CD42b, and CD61 [98]. Despite the fact that the abnormal cells are morphologically and immunophenotypically indistinguishable from acute megakaryoblastic leukemia (AML M7), they almost always disappear with little or no specific treatment. This process, known as transient abnormal myelopoiesis (also called transient leukemia or transient myeloproliferative disorder) is unique to babies with constitutional and mosaic trisomy 21. In the context of trisomy 21, acquisition of a truncating mutation in the GATA-1 transcription factor in a fetal liver-derived hematopoietic stem or progenitor cell results in expansion of the myeloid blast population. GATA-1 mutation is invariably present in DS babies with clinical TAM. Surprisingly, GATA-1 mutation was also detected in about 20% of DS infants without clinical evidence of TAM. These babies have so-called silent TAM [99].

The main clinical feature of TAM is leukocytosis with circulating blasts. There is currently no defined absolute blast threshold for the diagnosis of TAM. In a systematic review of 48 patients with TAM, the median age at diagnosis was 1 week, median WBC was about 30x10e9/L, and median peripheral blast percentage was 25%. Hepatosplenomegaly was present in more than half and liver dysfunction occurred in about one-third. Even though circulating blasts resolved in nearly all of the patients within a mean of 2 months, almost 20% of the patients died. Mortality in babies with TAM is most often attributed to hepatic fibrosis and liver failure [98]. Survival is associated with the absence of both hepatomegaly and life-threatening symptoms [100]. Treatment with very-low-dose cytarabine appears to decrease the risk of death in babies with severe TAM. However, chemotherapy treatment has never been shown to influence the risk of ML-DS later in childhood [101].

About 20% of TAM patients go on to develop myeloid leukemia before the age of 4 years. As in TAM, immunophenotypic markers of megakaryoblastic differentiation are observed in most cases and GATA-1 mutation is present in all cases. In association with evolution to ML-DS, additional "cooperating" mutations involving genes that encode cohesin complex components, epigenetic regulators, and/or signaling molecules are acquired [102]. In some children with ML-DS, a history of clinical TAM is absent. Presumably, these patients previously experienced undiagnosed "silent" TAM. Taking the incidence of silent TAM into account, it is estimated that progression to ML-DS occurs in about 5–10% of TAM cases [103] (Fig. 19.5).

ML-DS is a subtype of childhood AML that is clinically distinct and warrants distinct treatment. The median age at presentation is in the second year of life and the onset of the disease is often indolent, with gradual progression of cytopenias. ML-DS patients who do not meet the AML threshold of 20% blasts in blood or marrow often fulfill WHO criteria for MDS. In contrast to TAM, successful treatment of ML-DS requires chemotherapy. However, the intensity of chemotherapy necessary to cure ML-DS is less than de novo AML in children without DS. In fact, in an analysis performed by the Children's Cancer Group, higher intensity chemotherapy led to significant treatment-related toxicity in children with ML-DS, substantially offsetting the potential benefit in terms of leukemia control [57]. Since the late 1990s, treatment protocols designed specifically for children with ML-DS have progressively reduced treatment intensity while preserving good results with overall and event-free survival at around 80% [104–106]. Older age, higher presenting white blood cell count, and "normal" karyotype are associated with a significantly worse prognosis [107, 106].

Relapsed and Refractory AML

AML that is refractory to initial treatment or recurs after initial treatment represents a significant therapeutic challenge. Refractory disease occurs in about 5% and relapse affects about 30% [108]. Approximately half of relapses occur within 1 year, and almost all occur within 4 years of initial diagnosis [109, 110]. Long-term disease control can be accomplished in a minority of these patients but, in general, requires attainment of remission followed by HCT. For patients with relapsed AML, favorable cytogenetics [111], longer duration of first complete remission (i.e., ≥1 year from initial diagnosis), and receipt of HCT after relapse are associated with better outcomes [112].

There is currently no standard re-induction protocol for children with relapsed AML. Like up-front therapy, relapsed therapy mainly relies on the use of cytarabine with or without an anthracycline. Newer agents, including the purine analogues fludarabine, clofarabine, and cladribine, are incorporated into some regimens. Several combinations have been tested in children including fludarabine, cytarabine, idarubicin (Ida-FLAG) [113], mitoxantrone, cytarabine [114], fludarabine, cytarabine +/− liposomal daunorubicin [115], clofarabine, and cytarabine [116]. With any combination,

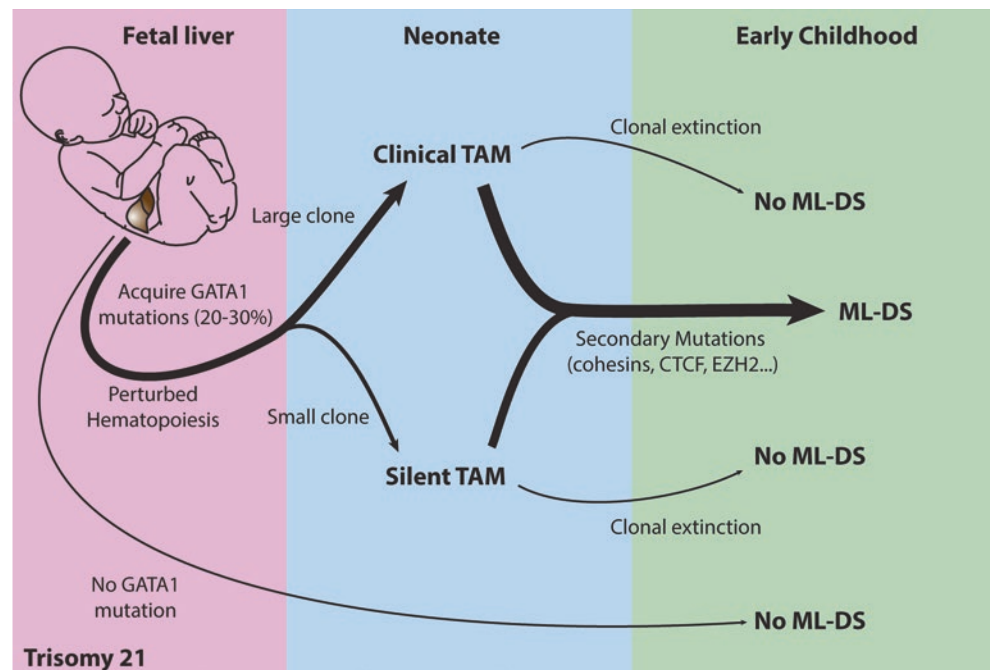

Fig. 19.5 Progression of transient abnormal myelopoiesis to Down syndrome-myeloid leukemia

complete remission is achieved in about 60–70% and many of these patients are able to proceed to HCT. MRD before HCT is a strong predictor of survival [117]. Both subsequent relapse and high treatment-related toxicity are major obstacles to overall survival. Treatment with novel and/or targeted agents, preferably in the context of clinical trials, may be an option for some patients.

The prognosis for patients with relapsed APL is much more favorable than for other relapsed AML subtypes. A role for autologous HCT has been demonstrated for those patients who achieve a second molecular remission [118]. In contrast, relapsed/refractory ML-DS is associated with a dismal prognosis [119].

Treatment Toxicity and Supportive Care

Improved survival in childhood AML is the product of maximizing treatment intensity in coordination with optimizing supportive care. Current treatment protocols appear to be reaching the inevitable limit of dose intensification. The severity and importance of treatment-related risks differ in children compared to adults. In general, children have fewer comorbidities and they tolerate myelosuppression better than adults. However, effects on growth and development matter much more. For children under 1 year of age and those who have a body surface area of less than 0.6 m^2, cytotoxic chemotherapy doses are adjusted, either as percent reduction or by basing calculations on weight instead of body surface area.

All children undergoing treatment for AML experience repeated episodes of prolonged and profound myelosuppression. Infections are the main cause of treatment-related morbidity and mortality. Unsurprisingly, the incidence of infection correlates with treatment intensity [120]. The use of bacterial and fungal prophylaxis is now recommended [121] as this does appear to reduce the rate of severe bacterial and invasive fungal infections [122]. In addition, prophylaxis against pneumocystis pneumonia is advised. The benefit of granulocyte colony-stimulating factor (filgrastim) remains controversial. When addition of prophylactic filgrastim was studied in a randomized fashion in the context of intensive chemotherapy in the Berlin-Frankfurt-Muenster (BFM)-98 study, the duration of neutropenia was shortened but the rate of severe infections was not reduced. However, in an analysis of the COG AAML0531 study, in which infections were collected and monitored prospectively, filgrastim prophylaxis was associated with a statistically significantly lower rate of bacterial infections [123]. However, its use is not without risk. A higher incidence of relapse was observed in children treated with filgrastim whose AML expressed a specific G-CSF receptor isoform [124]. At this time, routine use of filgrastim is not recommended.

Most children treated for AML receive substantial cumulative exposure to anthracyclines, often greater than 360 mg/ m^2. As a consequence, survivors are at high risk of long-term cardiac sequelae [125, 126]. Dexrazoxane has not been extensively studied in pediatric AML, but it might reduce cardiotoxicity [127]. Cardiotoxicity is a particular concern for children with DS [128]. After completion of treatment, patients should be monitored for cardiac late effects according to established guidelines [129].

Conclusions

Among pediatricians, it is often said that children are not just small adults. Although pediatric and adult AML share many features, there are important differences in terms of epidemiology, etiology, cytogenetics, and molecular genetics which influence therapy and outcome. Today, childhood AML is a curable disease in more than half of the cases. From the “glass is half-full” perspective, this fact unquestionably represents a major accomplishment. But, much more progress needs to be made to improve outcomes for all children and to reduce the burden of treatment. Progress is needed in many areas including molecular diagnostics, refinement of risk stratification, optimization of chemotherapy, development of targeted agents, and application of conventional and novel transplant strategies. Better outcomes also depend on advances in supportive care and implementation of best clinical practices. The way forward requires cooperation among pediatric oncology providers, scientists, and the pharmaceutical industry and on our patient’s ability to access and participate in well-designed clinical trials.

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Diagnosis and Treatment of Adult Acute Myeloid Leukemia Other than Acute Promyelocytic Leukemia

20

Peter H. Wiernik

Introduction

Acute myeloid leukemia (AML) includes all acute leukemias characterized by cells of other than lymphoid origin. AML subgroups with special clinical features have been defined by morphologic, immunologic, cytogenetic, and molecular techniques as discussed in Chaps. 14, 15, and 16. All subtypes other than acute progranulocytic leukemia (APL) are discussed in this chapter and APL is discussed in Chap. 21.

From a patient management point of view, the most serious pathologic consequence of AML is usually pancytopenia, rather than the production of leukemic cells. Therefore, management of AML requires prophylaxis and treatment of life-threatening complications of the absence of normal blood elements as well as eradication of the neoplastic clone from which the leukemic cells are derived. Prevention and treatment of the challenges to health posed by pancytopenia are discussed in the section "Supportive Care." It will be evident from those chapters that the management of a patient with AML is complicated and must be provided by a coordinated team of healthcare professionals thoroughly versed in the clinical nuances and complications of the disease, treatment of the disease, and impact of this catastrophic illness on patient, family, and society if optimal results are to be achieved. Such care is usually available only at major institutions and it is strongly recommended that, in general, the patient with AML be referred to such an institution immediately after the diagnosis is made. Some patients may not wish to be treated with curative intent and therefore need not be referred.

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Clinical Features of AML at Presentation

AML is diagnosed primarily in adults, although it can occur at any age. The median age at diagnosis in most large series is in the fifth or sixth decade, and the sexes have an approximately equal incidence. There is usually only a vague history of lethargy or lassitude prior to diagnosis, but approximately one-fourth of patients present with a serious infection of soft tissue or the lower respiratory tract associated on occasion with septicemia. Most patients have petechiae as evidence of intracutaneous capillary bleeding, but rarely more serious bleeding may be present initially. Bleeding gums after teeth brushing lead to the diagnosis in some cases. Lymphadenopathy is unusual in AML and splenomegaly is found in less than 25% of patients. If hepatomegaly is present it is almost always due to a cause other than AML in a de novo patient. Gingival hypertrophy (Fig. 20.1) is found in approximately half of patients with acute monocytic (FAB M5) or myelomonocytic (FAB M4) subtypes of AML. M4 and M5 patients have the highest incidence of all forms of extramedullary infiltration including leukemia cutis, and central nervous system (CNS) disease [1]. In some series granulocytic sarcoma is more common in patients with the M2 subtype of AML who demonstrate the t(8;21) cytogenetic abnormality. Perirectal lesions such as fissures or abscesses [2] may be present initially or during severe granulocytopenia at any time, especially in patients with M4 and M5 subtypes. The FAB subtypes defined by morphology and histochemistry, and the distribution of the subtypes among patients with AML, are identified in Table 20.1. More recent classifications based on cytogenetics and molecular characteristics are shown in Tables 20.2 and 20.3.

An elevated white blood cell (WBC) count is found in approximately one-third of patients with AML at diagnosis, and an equal number of patients have a normal WBC count or leukopenia. Hyperleukocytosis (WBC count >100,000 cells/μL) is uncommon, but may require special therapeutic interventions when present (see text to come). Blast forms

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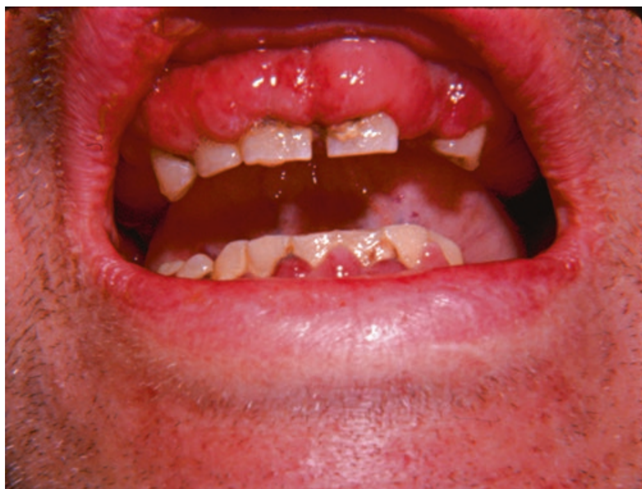

Fig. 20.1 Gingival hypertrophy in a patient with FAB M5 subtype of AML. Leukemic infiltration is the cause

Table 20.1 The French–American–British (FAB) Classification for AML

FAB type	Definition	% of adult AML patients
M0	Undifferentiated AML	5
M1	AML with minimal maturation	15
M2	AML with maturation	25
M3	Acute promyelocytic leukemia	10
M4	Acute myelomonocytic leukemia	20
M4E	Acute myelomonocytic leukemia with eosinophilia	5
M5	Acute monocytic leukemia	10
	(a) Monoblastic (b) Monocytic	
M6	Acute erythroid leukemia	5
M7	Acute megakaryocytic leukemia	5

Table 20.2 WHO Classification of AML with recurrent genetic abnormalities [3]

Karyotype abnormalities	Gene abnormalities
AML with t(8;21)(q22;q22)	RUNX1-RUNX1T1
AML with inv(16)(p13.1q(22) or t(16;16)(p13.1q22)	CBEB-MYH11
APL with t(15;17)(q22;q12)	PML-RARα
AML with t(9;11)(p22;q23)	MLLT3-KMT2A
AML with t(6;9)(p23;q34)	DEK-NUP214
AML with inv(3)(q21.3q26.2) or t(3;3)(q21.3;q26.2)	GATA2, MECOM
Acute megakaryoblastic leukemia with t(1;22)(p13.3;q13.3)	RBM15-MKL1
AML with	Mutated NPM1
AML with biallelic mutations of	CEBPA
Provisional: AML with	BCR-ABL1

Table 20.3 European LeukemiaNet Risk Stratification of AML [4, 5]

Risk groups	Included
Favorable	t(8;21); RUNX1-RUNX1T1
	Inv(16) or t(16;16); CBFB-MYH11
	Mutated NPM1 without FLT3-ITD and normal karyotype
	Biallelic mutated CEBPA and normal karyotype
Intermediate I	All cases with a normal karyotype except those in the favorable-risk group
	Wild-type NPM1 with or without FLT3-ITD
	Mutated NPM1 without FLT3-ITD
Intermediate II	t(9;11); KMT2A
	Cytogenetic abnormalities not favorable or adverse
Adverse	GATA2-MECOM (EVI1)
	T(6;9); DEK-NUP214
	T(v;11)(v;q23); KMT2A rearranged
	−5, or del 5(q); −7; abnl (17p)
	Complex karyotype without t(8;21), inv(16) or t(16;16), t(9;11) or other favorable abnormalities

are present in the peripheral blood of 85% of patients with AML before treatment. Therefore, about 15% of patients will not have a firm diagnosis made by examination of peripheral blood alone. The absolute granulocyte count is reduced in virtually all patients with AML and is less than 500 cells/ μL in approximately half of patients on the first examination. Thrombocytopenia is virtually universal and as many as one-third of patients will present with a platelet count <20,000/μL and they are candidates for immediate prophylactic platelet transfusion. Moderate anemia is the rule, but severe anemia may be found in patients with active bleeding other than petechial, or in patients in whom the diagnosis was delayed.

All patients with AML require bone marrow aspiration and biopsy. A biopsy is necessary to determine marrow cellularity. While the marrow is usually markedly hypercellular in de novo patients it may be hypocellular, especially in older patients, patients with secondary AML after treatment of another neoplasm with chemotherapy or radiotherapy, or patients who have developed AML after certain nonmalignant hematologic entities such as paroxysmal nocturnal hemoglobinuria [6]. Obviously, the marrow specimen must be obtained from a previously unirradiated site. It is important to assess marrow cellularity before and after treatment so that meaningful comparisons can be made. The pretreatment and subsequent marrow aspirates should be examined for morphology, histochemical reactions, immunophenotype, karyotype, and certain genetic mutations as discussed in this and other chapters in this section. Marrow aspirates submitted for immunological, cytogenetic, and molecular studies must be collected in heparin or acid citrate dextrose (ACD).

Leukemic blast cells account for at least one-half of marrow-nucleated elements in approximately 75% of AML patients at presentation. In elderly patients the leukemic cells may be less numerous. Usually, a diagnosis of AML is not made unless blasts account for at least 30% of the marrow white cells. Serial examinations in some patients will be necessary to determine the correct diagnosis and the rate of progression of the marrow infiltration. Rarely, the number of marrow blasts may increase slowly in some patients over several months or longer. It may be possible to withhold chemotherapy temporarily in some patients under those circumstances, especially elderly patients, as long as they are clinically well and the blood platelet and granulocyte counts are not dangerously low (<20,000/μL and <1000/μL, respectively).

The marrow aspirate may reveal other abnormalities in addition to leukemic cell infiltration. In patients with the M4 subtype relative erythroid hyperplasia is often present, despite anemia. There may be increased numbers of eosinophil precursors, especially in the M4E variant. Megakaryocytes are usually reduced in number except in secondary leukemia developing in a patient with polycythemia vera or primary thrombocytosis. Patients with the M7 subtype may have morphologically recognizable megakaryocytosis, but more often cell surface immunological or electron microscopic studies will be necessary to establish the lineage of the leukemic cells. Bone marrow necrosis may be evident prior to therapy [7], or discovered after therapy [8], especially in septic patients, and myelofibrosis may be detected in secondary leukemia or the FAB M7 subtype. Both marrow necrosis and myelofibrosis impair prognosis.

A minimal or moderate elevation in serum uric acid concentration is found in at least 50% of patients with AML. Serum lactate dehydrogenase levels may be elevated, especially in M4 or M5 subtypes, but usually to a lesser degree than in patients with acute lymphocytic leukemia (ALL). Lysozyme (muramidase) is elevated in the serum [9, 10] and urine of patients with M4 and M5 subtypes. As is the case with serum uric acid, levels of lysozyme directly reflect the body burden of tumor. Serial determinations of lysozyme may aid in evaluating response to therapy in patients with initial elevations [10].

AML is not simply a disease of the bone marrow and blood. Dysfunction of a number of organs may result directly from leukemic infiltration or indirectly from other consequences of the disease, and may dominate the clinical picture. Petechiae resulting from capillary hemorrhage secondary to thrombocytopenia are the most common skin and mucous membrane lesions. They tend to occur on dependent or traumatized areas of the body surface and may become confluent over some areas, especially in obese patients. Petechiae also occur on the surface and in the parenchyma of internal organs, but such lesions are usually clinically silent. Painless, nontender, small, raised nodules of leukemic cells may be palpable on the skin (leukemia cutis) of a small minority of patients with AML, especially those with M4 or M5 subtypes [11, 12], and approximately half of patients with leukemia cutis have an NPM1 mutation in their leukemia cells [13]. Such lesions are usually pink in color and not pruritic (Fig. 20.2). On rare occasion, leukemia cutis may be evident before bone marrow or other evidence of the disease is discovered [11], or it may be the first sign of relapse. Leukemia cutis has rarely been noted solely around central venous catheter exit sites or other injection sites [14, 15]. Leukemia cutis does not alter prognosis, but can be disturbing to the patient or even grossly disfiguring [16]. The lesions usually involve the entire corium, and the cells comprising them may have a different phenotype than the leukemic marrow cells [11]. The discordance may be due to partial differentiation of the skin lesion cells into macrophages [11].

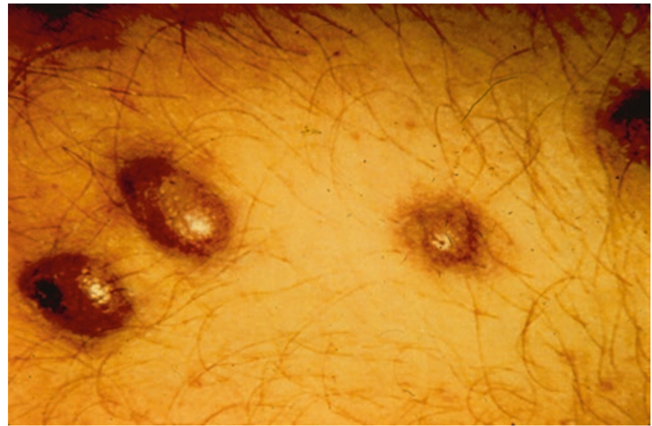

Fig. 20.2 Leukemia cutis in a patient with FAB M4 subtype of AML. The raised papules are due to leukemic infiltration of all layers of the corium

The lesions almost always respond to systemic chemotherapy rapidly, even if a complete remission is not ultimately obtained.

Rarely, a patient, especially a young patient, with AML will present with or develop a large subcutaneous or other mass of leukemic cells termed a granulocytic or myeloid sarcoma. On occasion, there is no other evidence of acute leukemia [17, 18]. Such lesions may also arise from subperiosteal areas of bone, particularly ribs, sternum, and orbit [19]. Granulocytic sarcoma of bone is rare, and other bone lesions such as radiographically seen metaphyseal lines that occur frequently in children with ALL are even rarer in adults.

Granulocytic sarcomas may occur in ovary [20], uterus [21], breast [22], cranial or spinal dura [23] (Fig. 20.3), and gastrointestinal tract [24] including liver [25], lung [26], mediastinum [27], prostate [28], and other organs and may present diagnostic difficulties in the absence of the usual

manifestations of AML [17, 29]. Such lesions may present as primary tumors of the organs involved, or suggest the diagnosis of lymphoma, plasmacytoma, or eosinophilic granuloma [30]. Typical AML may be discovered simultaneously, later, or never. A Wright-stained touch preparation of the lesion may help immeasurably in establishing the correct diagnosis. Immunohistochemical studies of fixed tissue may also be helpful in addition to routine histological studies [31, 32]. When isolated granulocytic sarcomas occur without other evidence of AML, radiotherapy [33] or surgery may be indicated. Although there is some evidence that treatment of an isolated granulocytic sarcoma with systemic chemotherapy will prevent the later occurrence of typical AML [34], this is not always the case and it is best to withhold systemic therapy until frank leukemia develops unless the granulocytic sarcoma cannot be treated locally.

Granulocytic sarcomas may occur more frequently in patients with M2 AML and t(8;21) [23, 35, 36], but it is clear that they also occur in patients with other cytogenetic abnormalities [37]. Their increased frequency as paraspinal tumors with t(8;21) may be related to the co-expression of a neural cell adhesion molecule (CD56) expressed by leukemic cells with that karyotype [38].

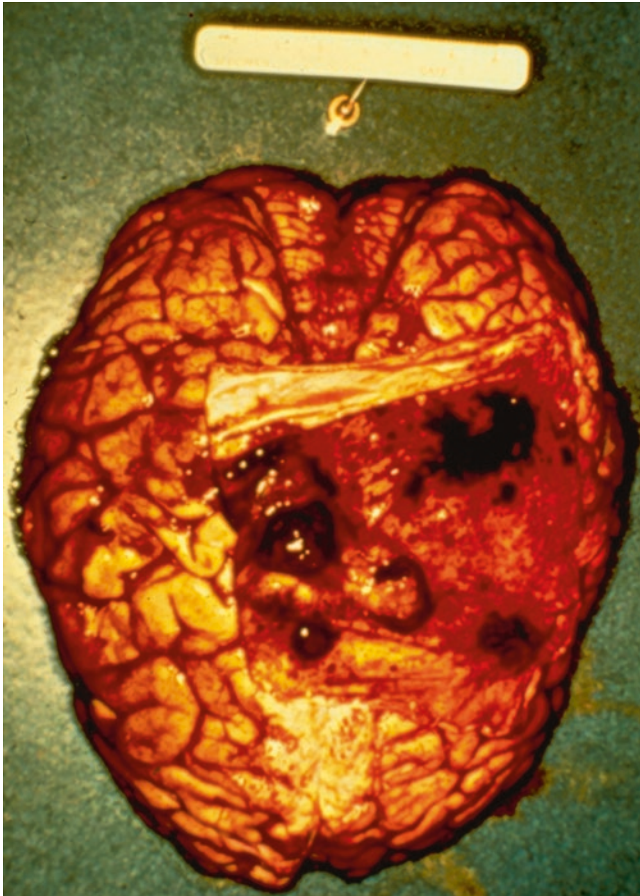

Fig. 20.3 A granulocytic sarcoma arising from the dura of the brain in a patient with FAB M2 subtype of AML with t(8;21) karyotype. The dura has been retracted to expose several dark nodules of tumor, which were *dark green*, due to myeloperoxidase contained in the cytoplasmic granules of the myeloid blast cells. The color fades when exposed to light

Acute febrile neutrophilic dermatosis (Sweet's syndrome) is a rare skin disorder that occurs in 1% of patients with AML for unknown reasons. It is more common in AML patients with FLT3 mutations [39]. The syndrome is characterized by fever, multiple painful papular and erythematous cutaneous eruptions, and a dense dermal infiltrate of mature granulocytes [40, 41]. A rapid response to glucocorticoids is usually obtained [42].

Fundic hemorrhage (Fig. 20.4) due to thrombocytopenia or leukemic infiltration of the retina may be found in patients of all ages with acute leukemia [43], including adults with AML [44].

Retinal leukemic infiltration is uncommon, is essentially confined to those patients with extreme hyperleukocytosis (blood blast count >200,000/μL), and is seen as one or more Roth-like spots with surrounding hemorrhage upon fundoscopic examination. Such lesions should be immediately irradiated if sight in the affected eye is to be preserved, but hemorrhage alone responds to successful platelet transfusion [44]. Other fundoscopic findings, such as cotton-wool spots; central vein obstruction; and vitreous, choroidal, or macular hemorrhage, are occasionally found [43, 44]. Certain treatments, such as high-dose cytarabine, may cause conjunctival and corneal pathology that results in impaired visual acuity [45]. The lesions resolve and normal visual acuity returns after discontinuation of the drug, and the problem can be prevented or attenuated with glucocorticoid ophthalmic drops administered during cytarabine treatment in most patients [46].

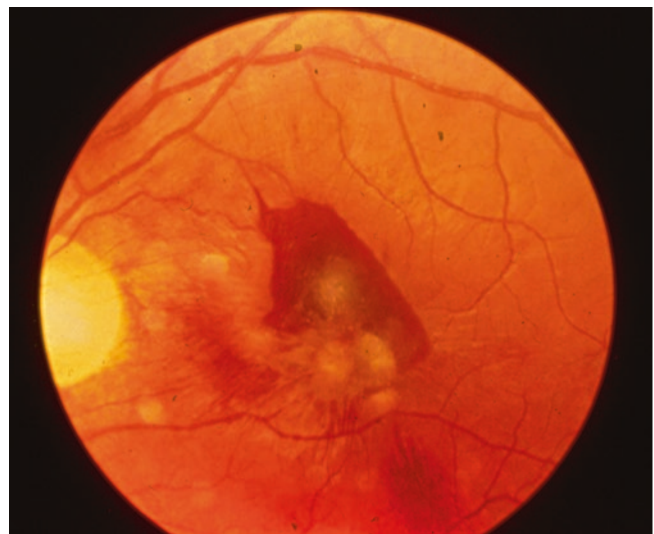

Fig. 20.4 Fundic hemorrhage in an AML patient with thrombocytopenia

Pulmonary dysfunction in patients with AML usually results from infection, which is discussed in Chaps. 53 and 54. A rare patient may develop dyspnea with or without an asthma-like syndrome due to pulmonary capillary leukostasis [47]. Such patients often have high blood blast counts [48, 49] and usually have the M3, M4, or M5 subtype of AML, but the frequency of this syndrome in patients with moderate degrees of leukocytosis may be underestimated [47]. The chest radiograph may be normal, show a ground-glass appearance suggestive of hemorrhage, or reveal diffuse alveolar consolidations [50]. This complication is frequently not recognized premortem, especially in patients with unrevealing chest radiographs [51]. Therefore, a therapeutic trial of bilateral low-dose whole-lung irradiation should be considered in an AML patient who has inexplicably developed progressively deteriorating pulmonary function [52–54]. A special form of this problem may occur after all-*trans* retinoic acid (ATRA) or arsenic trioxide therapy for acute promyelocytic leukemia and is fully discussed in Chap. 23. Severe and often fatal bilateral pulmonary hemorrhage may occur in end-stage patients who are thrombocytopenic and refractory to platelet transfusion, or in patients with a coagulopathy. This problem rarely arises during initial treatment. Although such hemorrhage has usually been ascribed to severe thrombocytopenia it may occur after successful platelet transfusion and other evidence suggests that its cause may be multifactorial. Diffuse alveolar cell damage may precede the hemorrhage, and cytoplasmic swelling and bleb formation have been noted in both capillary endothelial and alveolar lining cells in such patients [55]. While it is possible that these histologic changes represent toxic effects of extravascular blood, similar changes have resulted from cytarabine administration [56] or sepsis [57].

Heart conduction defects, murmurs, pericarditis, and congestive heart failure secondary to leukemic infiltration have been reported in AML [58–60]. Rarely, leukemic cardiac infiltration may occur in the absence of other evidence of AML [61]. These lesions are quite responsive to radiotherapy, which should be considered when leukemic infiltration of the heart cannot be ruled out [33].

It is important to have a dentist examine a patient with AML prior to therapy. Periodontal infections are common when AML is diagnosed and they may result in septicemia in a granulocytopenic patient. Dental extractions may be required [62] before initiation of chemotherapy, but often dental infections can be managed medically without interruption of leukemia treatment [63]. Other problems experienced by patients with AML in the region of the head and neck include leukemic infiltration of or hemorrhage into oropharyngeal structures that result in dysphagia or obstruction [64–66]. Leukemic infiltration of the inner, middle, and external ear has also been reported [67, 68].

As noted earlier, perirectal abscess and rectal fissure may develop in AML patients especially with the M4 or M5 FAB types. A small mucosal tear exquisitely painful on defecation or examination associated with fever may be the only indication of this potentially serious problem in a granulocytopenic patient, since infiltration and inflammation are often minimal [69]. Such lesions are usually the result of infection with gram-negative organisms, and bacteremia is frequent if proper treatment is delayed.

Necrotizing enterocolitis, or typhlitis, previously thought to occur primarily in children with acute leukemia, is described with increasing frequency in adults with AML who have been treated intensively [70]. Common symptoms include abdominal pain and distention with or without lower gastrointestinal bleeding. Abdominal radiographs may show only a nonspecific bowel gas pattern or lesions as serious as pneumatosis intestinalis, usually in the right colon. The lesions consist of mucosal ulcerations with inflammatory or leukemic infiltrates and usually involve the cecum but may also involve the ileum or the ascending colon. Bacteremia or fungemia frequently accompanies these lesions. Medical management may suffice [71], but surgery, which is usually successful when appropriate supportive care is available, may be required in some cases [72].

Renal dysfunction secondary to leukemic infiltration of the kidney or urate nephropathy is uncommon in adults with AML. Leukemic infiltration of the prostate [73] may obstruct the flow of urine and may rarely require irradiation. In most instances, however, induction chemotherapy will completely resolve the problem. Rarely, prostatic infiltration may be the first and only evidence of AML. Under no circumstances should a urinary catheter remain in place in a granulocytopenic AML patient.

Testicular relapse is common in ALL, especially in children, and has also been reported in adults with AML [74]. Postrelapse survival is frequently compromised in such patients.

Potassium wasting and other evidence of renal tubular dysfunction may occur in patients with the M4 and M5 subtypes who excrete lysozyme (muramidase), which is toxic to the proximal renal tubular epithelium [10]. The problem resolves with reduction of the tumor cell mass with chemotherapy. Lactic acidosis is a rare but difficult problem in AML [75]. Patients usually have large, vacuolated leukemic cells that may be difficult to accurately classify histologically. The etiology of the acidosis is obscure. Most patients have poorly controlled disease, and many have significant hepatic leukemic infiltration. The acidosis may require phenomenal quantities of alkali for control even after a partial remission of the leukemia is obtained.

Patients with AML may develop hypercalcemia [76], but hypocalcemia is more common. The latter may be a result of increased endogenous phosphorus production secondary to destruction of leukemic cells by either ineffective leukopoiesis, chemotherapy, or both, but septicemia and nephrotoxic antibiotics are frequently contributing factors [77]. On rare

occasion, hypocalcemia and hypophosphatemia may result from accelerated bone formation stimulated by leukemic cells [78].

Patients with AML subtypes M4 and M5 often present with hypocholesterolemia, which is thought to be due to increased low-density lipoprotein catabolism by mature monocytic phagocytes. Cholesterol levels return to normal with remission, and fall again with relapse of the leukemia [79].

Rarely, an AML patient presents with a markedly elevated peripheral blood blast cell count (>200,000 blasts/μL). This is a medical emergency since such a patient has approximately a 25% chance of a fatal intracerebral hemorrhage within a day or two [80–82]. This potential catastrophe is the result of intracerebral leukostasis secondary to increased blood viscosity. The hyperviscous blood causes sludging of blast cells at the low-pressure venous end of the capillary bed, which leads to plugging and eventual rupture of the vessel. The bleeding that then occurs would go unnoticed in most organs, but not in the brain. Those patients who undergo induction therapy with hyperleukocytosis are at risk for tumor lysis syndrome, which can be fatal even if recognized early [83]. Therefore, prophylactic emergency treatment directed at rapidly lowering the blood blast count and destroying established intracerebral foci of leukemic cells must be initiated at once (see discussion to come). A more common manifestation of CNS leukemia is meningeal infiltration with leukemia cells, which may arise from petechial hemorrhage in the meninges. Less than 2% of AML patients will have CNS leukemia at diagnosis. They are usually <45 years old, have a high WBC count of at least 50,000/μL, and have M4 or M5 FAB type (1). With proper treatment CNS leukemia at presentation does not impair prognosis. However, an isolated CNS relapse carries a poorer prognosis [1].

Many patients with AML are anergic to a battery of intradermal skin tests. This finding is of little clinical significance today since modern therapy has eliminated cutaneous anergy as a poor prognostic factor in AML. Some AML patients have decreased serum concentration of IgG and increased IgM concentration of unknown significance at presentation. Immunoglobulin levels usually normalize during induction therapy. On rare occasions, a serum paraprotein is present initially, which disappears after chemotherapy [84, 85]. Most patients with AML have a normal ability to raise a secondary antibody response [86, 87].

Diagnosis of AML

A thorough evaluation of a patient suspected of having AML must be conducted in a systematic fashion. A complete history should be taken with emphasis on exposure to medications, chemicals, and radiation, and the presence or absence of other diseases associated with an increased incidence of AML, including other neoplasms. A thorough family history should be taken, since a surprising number of patients with AML have a history of hematologic disorders in the family.

A complete physical examination is essential. If the patient is febrile, a thorough search for a focus of infection (periodontal disease, hemorrhoids, sinusitis, otitis, pharyngitis, pneumonia, abscess) must be made. The presence or absence of lymphadenopathy, splenomegaly, optic fundus pathology, CNS leukemia including cranial nerve palsy, and bleeding must be established. Granulocytopenic patients should not routinely undergo digital rectal examination.

Required peripheral blood studies include hematocrit, WBC count, platelet count, and differential WBC count. The peripheral blood smear should be examined by an oncologist or hematologist with experience in hematologic malignancies. A bone marrow biopsy and aspiration should be obtained from the posterior iliac crest with a Jamshidi needle or similar instrument. If it is impossible to obtain a posterior iliac crest aspirate, an attempt to obtain one from the sternum just under the ridge of the sternal angle with an Illinois or similar needle should be made. It is important to learn to perform these procedures properly from someone with experience. The biopsy is necessary to determine marrow cellularity and to assess the extent of the leukemic infiltrate. The aspirate should be examined after thin air-dried preparations are made, preferably on cover slips. No anticoagulant should be added to the aspirate obtained for routine staining and histochemistry, since some anticoagulants cause morphologic abnormalities in the leukemic cells, such as vacuolization, which may lead to diagnostic confusion. Aspirate smears should be stained with Wright's stain and a battery of histochemical reactions as detailed in Chap. 16. Such stains facilitate differentiation among the various AML subgroups, and between AML and ALL, and are required for proper French–American–British (FAB) classification. An iron stain should also be obtained on the biopsy to assess iron stores, and on the aspirate to identify sideroblasts often found in secondary AML, especially after treatment for Hodgkin's disease or multiple myeloma, and ringed sideroblasts that may be found in erythroleukemia (FAB M6).

An aspirate anticoagulated with heparin or ACD should be sent for immunophenotypic, cytogenetic, and molecular studies. The importance of these studies in the diagnosis of AML is detailed in Chaps. 17 and 18, and may give important prognostic information as discussed below.

Certain blood chemistry studies are required for proper assessment of the patient. Serum electrolytes, uric acid, lactate dehydrogenase, creatinine, lysozyme, and blood urea nitrogen should be determined. Routine coagulation studies and a plasma fibrinogen concentration are especially important in a patient suspected of having the M3 subtype of AML. Since hypogranular variants of that subtype exist, it is

important to study all patients initially. It should be remembered that some antibiotics commonly used in leukemia patients may cause abnormalities of coagulation unless vitamin K is administered prophylactically.

It is only necessary to examine the cerebrospinal fluid (CSF) routinely in asymptomatic AML patients with the M4 subtype. A lumbar puncture should only be performed in thrombocytopenic patients after a successful platelet transfusion has elevated the platelet count to 75,000/μL or more and in patients with a coagulopathy only after the plasma fibrinogen level has risen above 100 mg percent. Only 25-gauge needles should be used. The CSF obtained should be studied for routine parameters and, in addition, a cytocentrifuged specimen should be studied after staining with Wright's stain. Some training is required to accurately assess such specimens. Occasionally ependymal and other cells will be seen that may be mistaken for leukemic cells by the untrained observer. An elevated β_2-microglobulin CSF concentration may suggest occult CNS leukemia [88].

A posteroanterior and lateral chest radiograph should be obtained primarily as a baseline in an asymptomatic patient. Rarely, a mediastinal mass will be observed. This finding may confuse the observer unless one is aware of this rare manifestation of granulocytic sarcoma in AML [27].

Finally, the patient's blood should be typed and at least two packed red cell units cross matched with the patient's blood should be available at all times. If the patient has circulating lymphocytes the HLA type should be determined so that this information is available if bone marrow transplantation is contemplated in the future or if HLA-compatible platelet transfusions become necessary. At the same time, family members who agree to donate platelets, granulocytes, or bone marrow to the patient should also be HLA typed.

Preparation for Induction Therapy

It may not be necessary to begin induction chemotherapy immediately upon the diagnosis of AML. It is best to spend a day or two diagnosing the leukemic disorder precisely and resolving whatever medical emergencies are evident or developing.

Thrombocytopenic hemorrhage is more easily prevented than treated. Therefore, an AML patient with a platelet count less than 15,000–20,000/μL is a candidate for prophylactic platelet transfusion, which is discussed fully in Chap. 57. Prophylactic platelet transfusion has virtually eliminated hemorrhage as a cause of death during induction therapy. Platelet transfusion should not be given to a patient with a coagulopathy until low-dose heparin therapy is begun, or the coagulopathy may be aggravated.

An AML patient with a serious, uncontrolled infection at the time induction therapy is begun has a greatly reduced chance of remission. Therefore, documented or suspected infection should be under treatment and showing clear evidence of resolution before the institution of chemotherapy whenever possible. It is especially important not to begin chemotherapy until infection is controlled if the patient has circulating granulocytes. If absolute granulocytopenia exists in an infected patient, chemotherapy and antibacterial antibiotic therapy should be started simultaneously. Empiric broad-spectrum antibiotic therapy should be instituted immediately in a febrile granulocytopenic AML patient [89]. It should be remembered that fever may be the only clue to a serious infection in such a patient since the usual signs and symptoms of infection, which are largely due to granulocytic infiltration of infected tissues, may be absent [90]. Infection prevention and treatment for patients with AML are fully discussed in Chaps. 52 and 53, respectively.

The prophylaxis of intracerebral hemorrhage secondary to hyperleukocytosis [91] usually consists of emergency irradiation to the entire cranium with 600 cGy in a single dose and the administration of oral hydroxyurea (3 g/m^2 given daily for 2 days). The former will resolve already established intracerebral foci of leukemia, and the latter will rapidly reduce the blood blast count and thereby reduce blood viscosity, which is necessary to prevent reformation of intracapillary collections of blasts. Emergency leukapheresis has also been reported to be effective in this setting [92]. The procedure requires the availability of a blood cell separator and has not been demonstrated to be more effective than simple hydroxyurea administration. Management of hyperleukocytosis solely with hydration, urinary alkalinization, and allopurinol has been reported to be effective in infants [93] but is not recommended for adults.

Urate nephropathy is unusual in AML, except in patients with hyperleukocytosis or organomegaly due to leukemic infiltration. However, it is prudent to begin allopurinol (300 mg orally, daily for 1 or 2 days) before induction therapy and equally prudent to discontinue the drug after the marrow has become hypocellular following chemotherapy. Unnecessary prolongation of allopurinol administration may result in cutaneous eruption, which occurs with about 20% of prolonged courses of the agent or, on rare occasion, permanent marrow aplasia [94]. Patients who present with elevated serum uric acid concentration and an unusually large tumor load due to hyperleukocytosis or granulocytic sarcoma will require double or triple the usual allopurinol course initially, or treatment with recombinant urate oxidase (rasburicase) [95, 96].

Infection prevention methods should be instituted before induction therapy. The patient should be placed in strict reverse isolation in a meticulously cleaned room with air supplied only through high-efficiency particulate (HEPA) air filtration systems.

A triple-lumen Hickman catheter or similar device should be installed prior to treatment to facilitate blood drawing and intravenous therapy. If at all possible, the catheter should be placed at a time when the patient has circulating granulocytes, and use of the catheter should be restricted to personnel who have been specifically trained in the proper use and care of such devices.

Special consideration needs to be given to the pregnant patient with AML. Commonly administered induction agents other than idarubicin [97] can be given with relative safety to mother and fetus during the third and probably the second trimester [98–100] and should be given at doses based on actual body weight [101]. Children born to mothers undergoing induction therapy for AML during those trimesters have experienced only minor problems at birth and after long-term follow-up [98–102]. However, the use of lipophilic idarubicin during pregnancy may result in neutropenia and/or cardiac dysfunction in the neonate [97]. Induction therapy during the first trimester is very likely to result in abortion [99], and the diagnosis of AML itself in the first trimester may cause spontaneous pregnancy loss [101]. It may therefore be prudent to induce abortion under controlled circumstances in the first trimester. Rarely, spontaneous temporary remission of untreated AML may occur after cesarean section [22], or other event, usually a pyogenic infection [103].

Chemotherapy for AML

Chemotherapy for AML is administered in two stages: induction therapy followed by consolidation therapy. Allogeneic or autologous bone marrow transplantation may follow consolidation therapy in some circumstances, or consolidation therapy may be followed by or replaced by long-term maintenance therapy in other circumstances. The latter approach is not commonly used, although there is a rationale for it [104–106]. The purpose of induction therapy is to achieve complete clinical and hematological remission, which is defined as the absence of all clinical evidence of leukemia as well as a normocellular marrow devoid of leukemic cells and with normal trilineage hematopoiesis. Peripheral blood counts and differential WBC count are usually within the normal range in patients in complete remission, although in a minority of cases the platelet count may not recover to normal levels [107, 108]. Patients without complete platelet recovery may have impaired long-term survival compared with others [108, 109]. In patients whose AML is characterized by a specific gene mutation, quantitation of that mutation in marrow or peripheral blood cells [110] after complete hematologic remission is obtained may yield significant prognostic information with regard to the likelihood of relapse and even drive postremission therapy decisions. Such minimal residual disease testing is likely to become standard in the near future, once technical details are worked out [111–113]. The purpose of postremission therapy is to reduce the body burden of subclinical leukemia to, theoretically, zero. There is overwhelming evidence to support the concept of postremission therapy in that in virtually all studies in which outcome with and without postremission therapy has been prospectively compared, disease-free and overall survivals are greater in patients who continue treatment while in complete remission. Furthermore, most available data demonstrate a dose–response relationship for postremission therapy so that, in general, cure rates are higher with postremission dose-intense regimens than with regimens of lesser dose intensity. While there is no question that intensive postremission therapy is currently necessary in order to achieve optimal results, some studies have suggested that intensification of induction therapy may improve disease-free and overall survival despite no improvement in remission rate [114].

Although there is little evidence that the major FAB subtypes respond differently to standard induction therapy for AML, the development of ATRA therapy for the M3 subtype suggests that more subtype-specific therapy for AML may be developed in the future and that remission induction by mechanisms other than leukemia cell kill may be possible. There is already evidence that some dose-intense postremission regimens may be more beneficial in AML patients with favorable cytogenetics than in others [115].

There is no need for CNS prophylaxis in adult AML. The frequency of overt CNS leukemia is less than 1–2%, and cytarabine is virtually always used during induction therapy in intravenous doses that result in therapeutic CSF levels.

Results of induction therapy vary depending on a variety of prognostic factors. Patient age is the oldest recognized such factor. Patients over the age of 60–65 years have significantly lower complete response rates to induction therapy in most studies. Cytogenetic abnormalities are divided into favorable, intermediate, and unfavorable groups with respect to prognosis for complete response to therapy, and overall survival. The favorable group includes inv(16), t(8;21) without kit mutations, and t(15;17). A normal karyotype has an intermediate prognosis as does t(8;21) with kit mutation, and monosmy 5 or 7 (usually seen in secondary AML) as well as a complex karyotype have an unfavorable effect on prognosis [115]. See Tables 20.2 and 20.3.

More recently a number of genetic aberrations that affect prognosis in AML patients treated with currently available therapy have been identified [116]. In some instances, new treatments have been devised that partially offset the poor prognosis associated with some of these mutations. FLT3 mutation is one of the most common mutations seen in AML. The fems-like receptor tyrosine kinase (FLT3) expressed by immature

hematopoietic cells is important for the normal development of hematopoietic stem cells. Activating mutations caused by either an internal tandem duplication (ITD) or multiple amino acids in the juxtamembrane region or point mutation in the activation loop of the tyrosine kinase domain (TKD) are present in approximately 30% of patients with de novo AML. FLT3-ITD mutation is the most common molecular abnormality associated with adult AML. FLT3-ITD mutation occurs in patients with all FAB and cytogenetic designations, but is most common in patients with FAB M3. Such mutations have a negative impact on disease-free and overall survival in patients with a normal karyotype but have little influence on prognosis of patients with favorable or unfavorable cytogenetics. Recently developed inhibitors of these mutations given with standard induction therapy partially neutralize the activity of FLT3 mutations in the laboratory [117, 118] and partially negate the impaired prognosis conferred by these mutations in the clinic [119–121].

A number of other molecular prognostically significant factors have recently been identified, such as NPM1, CEBPA, IDH1, IDH2, and WT1 mutations, and they have the potential for becoming therapeutic targets in the future. NMP1 is frequently mutated in AML and preclinical studies suggest that cells with the mutation may undergo apoptosis with retinoic acid or arsenic trioxide treatment [122]. In a study of 148 AML patients 60 years old or older with normal cytogenetics, Becker et al. [123] reported that 56% of the patients had NPM1 mutations and those patients had a higher complete response rate [84% vs. 48% for patients without the mutation ($p < 0.001$)] as well as significantly longer disease-free and overall survival. The prognostic impact of the mutation was observed predominantly in patients at least 70 years old. Others have reported similar results [124]. See Table 20.4.

Damm et al. [125] found that patients with normal cytogenetics and a single-nucleotide polymorphism located in the mutational hot spot of the WT1 gene had improved relapse-free and overall survival compared with others, and Dufour et al. [126] reported that patients with normal karyotype biallelic CEBPA gene mutations, compared with those with monoallelic mutations or wild-type CEBPA, had significantly better overall survival after standard therapy. Conversely, Kornblau et al. [127] reported that highly phosphorylated Foxhead transcription factor (FOXO) in leukemic cells is a significant negative prognostic factor for survival in AML, independent of karyotype. Phosphorylated FOXO levels were higher in patients with FLT3 mutations, and were associated with higher WBC counts and a higher percent of blood and marrow blast cells.

In general, approximately 65–70% of unselected patients with de novo AML will achieve complete remission after one course of induction therapy. At least 30%, and perhaps as many as 40%, of complete responders will be cured after appropriate postremission therapy. In some studies long-term results are significantly better in women [128] and in virtually all studies they are better in patients <60 years old. Results of induction therapy are likely to improve as inhibitors of diver mutations are developed.

An excellent review of the molecular biology of AML has recently appeared [129].

Table 20.4 Examples of gene alterations affecting prognosis in AML [111, 129–135]

Gene	Prognostic effect	FAB type
FLT3-ITD	Poor with intermediate karyotype	
RUNX1	Poor	43% in M0
TP53	Poor-almost always with complex karyotype	36% in M6
FOXO phosphorylated	Poor	
EVI1	Poor	
IDH1, IDH2	Conflicting data	
DNMT3A	Poor if normal karyotype	26% in M2
KIT	Poor in CBF AML	
MLL-PTD	Poor	
TET2	Poor with intermediate karyotype	
TET2 hypomethylation	Very favorable	
NPM1	Favorable in FLT3-ITD mutation negative	42% in M1 57% in M4 49% in M5a 70% in M5b
CEBPA biallelic	Favorable	
WT1	Favorable if other than normal karyotype Unfavorable with normal karyotype	

PTD partial tandem duplication, *CBF* core-binding factor

Induction Therapy

The standard induction regimen for adults with AML for decades has been the two-drug combination of daunorubicin and cytarabine, and complete response rates on the order of 65% in unselected patients have regularly been reported with that combination [136–140] in unselected patients. Patients over the age of 60 years usually have a lower response rate. In a prospective, randomized ECOG study elderly patients treated with a standard induction regimen plus GM-CSF had a higher response rate, lower rate of infection, and lower death rate [141]. However, a similar study utilizing an investigational GM-CSF derived from *Escherichia coli* showed no advantage for the growth factor [142]. The ECOG study demonstrated that GM-CSF does not stimulate leukemia when used after marrow hypoplasia occurs, since patients receiving the growth factor did not have shorter disease-free or overall survival [141].

A commonly used induction regimen is a continuous intravenous 7-day infusion of cytarabine given at the rate of 100 mg/m^2 per day, plus daunorubicin given as 3 daily bolus injections of 45 mg/m^2 each, beginning on the first day of treatment. However, recent data suggest that much larger doses of daunorubicin may be more efficacious than standard doses. Fernandez et al. [143] randomly allocated 657 patients with previously untreated AML aged 17–60 years to cytarabine, 100 mg/m^2/day as a continuous 7-day I.V. infusion plus daunorubicin 45 mg/m^2 daily for 3 days or daunorubicin 90 mg/m^2 on the same schedule. The results were recently reported after more than 80-month median follow-up [144]. The higher daunorubicin dose resulted in a higher complete response rate (71% vs. 59%) and improved overall survival (median 25.4 months vs. 16.6 months, respectively). Only patients aged <50 years benefited from the high-dose therapy. The median overall survival with high-dose daunorubicin was 44.7 months compared with 20.7 months for the standard-dose patients younger than age 50. Patients with favorable cytogenetics benefited the most from the high-dose regimen but those with intermediate and unfavorable cytogenetics may have done so as well, but his could not be confirmed on univariate analysis. Patients with FLT3-ITD, NPM1, IDH, or DNMT3A mutations benefited as well from high-dose daunorubicin, and the high-dose induction regimen was required for the favorable impact of the NPM1 mutation on the disease to be evident. The rates of serious adverse events were similar in the two groups, but longer follow-up of survivors will be needed before that fact can be verified. Löwenberg et al. [145] conducted a similar study in older patients. They randomized 813 newly diagnosed AML or high-risk refractory anemia patients aged 60–83 years to receive daunorubicin at one of the two doses used in the Fernandez study [143] and cytarabine, 200 mg/m^2/day in a continuous 7-day infusion. The CR rate was 64% with the 90 mg/m^2 daunorubicin dose and 54% with the 45 mg/m^2 dose. Overall survival was similar in the two groups, but patients aged 60–65 years had greater event-free (29% vs. 14%) and overall (38% vs. 23%) survival with the 90 mg/m^2 dose of daunorubicin. A Korean study using the same regimens reported a higher complete response rate, and longer event-free and overall survival with similar toxicity in 383 patients ≤60 years old [146]. These three studies are interesting, but they need to be viewed critically. In many published studies the response rates with standard-dose daunorubicin are better than those reported in at least one of these studies [143] and similar to the results obtained with the daunorubicin 90 mg/m^2 dose. Patients who receive a total of 270 mg/m^2 daunorubicin with one course of the high-dose daunorubicin will probably not be able to receive retreatment in the future with an anthracycline should the need arise. Furthermore, the long-term toxicity of the high-dose daunorubicin regimen is unknown.

Burnett et al. [147] randomized 1206 patients, mostly younger than 60 years, with previously untreated AML or high-risk myelodysplastic syndrome to receive daunorubicin, 90 or 60 mg/m^2 on days 1, 3, and 5, combined with cytarabine, 100 mg/m^2/day as a 10-day continuous infusion. All patients received a second induction course with daunorubicin, 50 mg/m^2 on days 1, 3, and 5. There was no difference in complete response rate overall or 2-year overall survival in any subgroup. However, 60-day mortality was significantly increased in the patients who received daunorubicin, 90 mg/m^2. Although there are differences in the design of this study and the Fernandez study described above [143, 144] the results of the two studies are completely at odds with each other, especially with respect to toxicity of daunorubicin, 90 mg/m^2. Other smaller studies have addressed the question of daunorubicin dosage intensification. Prebet et al. [148] concluded from a retrospective study of AML newly diagnosed patients with core-binding factor that relapse-free survival was significantly better with daunorubicin 90 mg/m^2 than with daunorubicin 60 mg/m^2 and that there was a trend ($p = 0.07$) for a superior 2-year overall survival with the former. Another small retrospective study concluded that daunorubicin 90 mg/m^2 improved overall survival compared with daunorubicin 60 mg/m^2 [149]. Pautas et al. [150] compared daunorubicin 80 mg/m^2 daily for 3 days with idarubicin 12 mg/m^2 daily for 3 days or, in another group, daily for 4 days in 468 patients with ages ranging from 50 to 70 years. All patients received cytarabine as well. Both idarubicin schedules resulted in a significantly higher complete response rate than did daunorubicin, but there was no difference in relapse rate, event-free survival, or overall survival among the treatments.

From a meta-analysis of six randomized controlled trials Gong et al. [151] found significant improvement in complete response rate, event-free survival, and overall survival with daunorubicin 90 mg/m^2 compared with lower daunorubicin doses, but no differences in disease-free survival, relapse rate, or toxicity. Another meta-analysis found that both high-dose daunorubicin (90 mg/m^2 × 3 and 50 mg/m^2 × 5 studies lumped together) and idarubicin, 12 mg/m^2 × 3, achieve 5-year survival rates of 40–50% in patients ≤60 years of age [152]; another such study in adults showed that both high-dose daunorubicin and standard-dose idarubicin were superior to standard-dose daunorubicin in achieving complete response and long-term survival [153].

Dose intensification of daunorubicin during induction is not a new idea. Greene et al. [154] studied daunorubicin as a single dose of 180 mg/m^2 in 1972. Only a 25% complete response rate was obtained in 16 previously untreated patients aged 26–73 years. Seven of the 16 patients died during induction therapy and the rest suffered unacceptable toxicity.

Taken together, these data indicate that daunorubicin, 90 mg/m^2/daily × 3, is superior induction therapy for AML patients <60 years of age, compared with 45 mg/m^2. What is not clear is whether the higher dose is better than daunorubicin 60 mg/m^2 or a standard dose of idarubicin [155].

Daunorubicin, standard dose, and cytarabine were prospectively compared in six major randomized studies with an identical regimen except for the substitution of idarubicin, standard dose, for daunorubicin [156–162]. There were no significant differences in toxicity between the two treatments in any of the studies. In three of the studies [157–159] the complete response rate was superior with idarubicin plus cytarabine and the differences were significant for patients under the age of 60 years, and disease-free and overall survivals were significantly greater in idarubicin-treated patients in three of the studies [157, 158, 161]. The idarubicin–cytarabine regimen was significantly more effective in remission induction in patients with hyperleukocytosis than the daunorubicin–cytarabine regimen in the two studies in which that question was examined [157, 158]. In an Italian study for patients over the age of 55 years no difference in response rate or duration or survival was noted between the two treatments, but a significantly greater number of complete responders achieved remission with one course of idarubicin and cytarabine than with daunorubicin and cytarabine [161] and in a Japanese study no differences in outcome were noted [162]. In the ECOG study [156] of 349 patients over the age of 55 years the complete response rates were 40%, 43%, and 43% with the daunorubicin, idarubicin, and mitoxantrone regimens, respectively, and the differences were not significant. The median disease-free survival was 5.7, 9.7, and 6.9 months, respectively, but again the differences were not significant. A recent meta-analysis reported that idarubicin in induction therapy prolonged overall survival and disease-free survival, increased the complete response rate, and reduced the relapse rate compared with daunorubicin, although toxicity was greater with the former [163]. For patients under the age of 70 years the differences in complete response rates were greater (46%, 53%, and 52%, respectively), but the differences were still not significant. These data taken together strongly suggest that idarubicin is a more effective anthracycline than daunorubicin in the treatment of adult AML, especially in younger patients, and this fact was confirmed by a meta-analysis of 1052 patients randomized to receive daunorubicin or idarubicin, both at standard dosing, with cytarabine [164]. These clinical observations are consistent with the more favorable clinical pharmacokinetics of idarubicin [165] compared with those of daunorubicin, and with the observation that the intracellular accumulation of idarubicin is decreased to a much lesser degree by P-glycoprotein than that of daunorubicin [166].

Many investigators interpret currently available data to suggest that idarubicin should replace daunorubicin in the treatment of adults with AML, and an appropriate treatment regimen is detailed in Table 20.1.

In three randomized, prospective large studies the combination of mitoxantrone and cytarabine was compared with daunorubicin and cytarabine for induction therapy in adults with AML [156, 167, 168]. The standard dose and schedule of cytarabine were employed and mitoxantrone 12 mg/m^2 given daily for 3 days was substituted for daunorubicin in one arm of each study. No significant difference in outcome with respect to complete response rate, disease-free or overall survival, or toxicity was observed in any of the studies.

The addition of etoposide to the standard daunorubicin and cytarabine regimen improved disease-free and overall survival without improving the response rate, especially in patients less than 50 years of age in one study but not in others [169, 170].

Holowiecki et al. [171] reported that the addition of cladribine to a standard daunorubicin and cytarabine induction regimen increased the complete remission rate and overall survival at 3 years compared with the two-drug regimen in 652 newly diagnosed patients with AML ≤60 years of age. The survival advantage for the three-drug regimen was noted in patients ≥50 years of age, those with hyperleukocytosis, and those with unfavorable cytogenetics. These results deserve confirmation.

Hills et al. [172] performed a meta-analysis of studies in which gemtuzumab ozogamicin 3 mg/m^2 or 6 mg/m^2 was added to standard induction therapy or not, for adults with AML, and found that the agent prolonged survival at 5 years in patients without unfavorable cytogenetics. Both doses resulted in the same outcome but the lower dose was less toxic, as found in other studies [173]. This agent is no longer available in the USA but it clearly deserves further study.

Zeidner et al. randomized 165 newly diagnosed patients aged 18–70 years with intermediate or poor cytogenetics to cytarabine, 100 mg/m^2/day, as a continuous intravenous 7-day infusion plus daunorubicin, 90 mg/m^2, or a cyclin-dependent kinase inhibitor, alvocidib (formerly known as flavopiridol), together with cytarabine and mitoxantrone (FLAM). The complete response rate with FLAM was 70% compared with 46% for high-dose daunorubicin and cytarabine [174]. Further study of alvocidib in AML is planned.

Ravandi et al. [175] studied 62 patients with previously untreated AML with a median age of 53 years with the FLT3-ITD inhibitor sorafenib, cytarabine, and idarubicin. FLT3 mutations were present in 23 patients and 10 had unfavorable cytogenetics. A complete remission was obtained by 79% and an additional 8% attained a complete remission with incomplete platelet recovery. Interestingly, a 95% complete response rate was achieved in the patients with FLT3-ITD mutations. With a median follow-up of 52 months, the median survival for all patients was 29 months. Although this was a small study, it clearly suggests that sorafenib in

patients with FLT3-ITD mutations deserves further study. Other inhibitors of FLT3-ITD have yielded impressive results as well [176, 177].

High-dose cytarabine in induction therapy has been evaluated in a number of studies. In two early studies it was associated with greater toxicity than standard-dose cytarabine but there was no improvement in complete response rate or survival [178, 179] and a meta-analysis involving 5945 patients concluded that high-dose cytarabine led to a lower relapse rate than did standard-dose cytarabine but no improvement in complete response rate or overall survival [180]. In a more recent study, high-dose cytarabine produced a higher complete response rate and better overall survival especially in patients younger than 46 years and in patients up to 60 years of age with unfavorable cytogenetics or FLT3-ITD mutation [181]. In the most recent study [182] no advantage for high-dose cytarabine in induction was observed. High-dose cytarabine during induction is not recommended.

The addition of sorafenib, a tyrosine kinase inhibitor, to standard induction therapy led to significant improvement in event-free and overall survival after a median follow-up of 36 months, compared with placebo in one study of 267 evaluable patients ≤60 years [183]. Grade 3 or 4 toxicity was significantly higher in the sorafenib group, however.

Toxicity of Induction Therapy

Virtually all patients treated with the regimen recommended in Table 20.5 will develop total capital alopecia, which is often more disconcerting to young men than to others. Attempts to prevent alopecia with scalp tourniquets or hypothermia caps are ill advised during leukemia treatment since the scalp may become a pharmacologic sanctuary when such methods are employed.

Moderately severe nausea and vomiting accompanies induction therapy in approximately 80% of patients not premedicated with antiemetics. Older patients tend to have a lower incidence of emesis, perhaps due to poorer blood supply to the chemoreceptor trigger zone of the brain. Modern antiemetics, such as ondansetron [185] and granisetron [186], usually completely eliminate vomiting during induction therapy. It is not necessary to include dexamethasone in the antiemetic regimen, and it may indeed be unwise to do so since glucocorticoids inhibit anthracycline reductase activity, which may result in decreased anthracycline effectiveness.

Table 20.5 A standard remission induction regimen for adult AML

A. Idarubicin (12 mg/m^2) is given daily on each of the first 3 days of treatment. The drug is given as an injection over 10–15 min into a central venous catheter. Severe paravenous tissue damage may result from extravasation. Consider daunorubicin 60 mg/m^2 on the same schedule as an alternative anthracycline for patients <60 years old. Consider oral hydroxyurea [184] or leukapheresis for patients with a WBC > 50,000/μL prior to initiation of induction therapy
B. Cytosine arabinoside is given as a continuous 7-day intravenous infusion at the rate of 100 mg/m^2 per day beginning on the first day of idarubicin administration. The infusion must be controlled by an electronic device to ensure the proper rate of administration
C. Consider adding a FLT3-ITD inhibitor for patients with that mutation and intermediate cytogenetics

Stomatitis, esophagitis, and diarrhea are usually only of grade 1–2 intensity and can be expected in approximately 65%, 15%, and 80% of patients, respectively [158]. Oral mucosal ulceration is usually well managed with viscous xylocaine or a paste of gelatin, pectin, and carboxymethyl cellulose. Hepatic toxicity manifested by serum liver enzyme elevations occurs in half of patients and is usually unaccompanied by clinically significant hepatic dysfunction and virtually always resolves with the completion of induction therapy.

A generalized mild-to-moderate erythroderma may result from cytarabine or idarubicin treatment, and a unique cutaneous eruption has been reported after etoposide administration [187].

Profound bone marrow hypoplasia and pancytopenia are expected and desirable after induction therapy since, except in patients with the M3 subtype, complete remission is virtually never achieved without these results of therapy. Pancytopenic patients will require platelet transfusion and, very likely, packed red cell transfusion until the bone marrow recovers, usually in 3–4 weeks after the end of treatment. Transfusion support is discussed in Chaps. 56 and 57.

Response to Induction Therapy

Approximately 90% of patients who achieve complete remission will do so within the month after completion of the first induction course. Another 10% of patients who ultimately obtain a complete remission will require a second induction course to do so. The second course should be given in the same doses and schedule as the first. Patients who fail induction therapy with two courses of idarubicin and cytarabine may be candidates for regimens employing mitoxantrone [188], carboplatin [189], 2-chlorodeoxyadenosine [136], fludarabine [137], high-dose cytarabine [138], gemtuzumab ozogamicin [190], an investigational agent, or bone marrow transplantation, all of which are discussed next. Unfortunately, patients who fail initial therapy are not likely to subsequently do well, and at the present time it is difficult to recommend a standard approach to such patients [194]. Clinical trials should always be made available to patients who fail standard induction therapy.

Often the first sign of complete remission after induction therapy is a spontaneous rise in the platelet count. If the

marrow aspirate demonstrates repopulation with normal elements at that time, and leukemic cells are rare, blood counts should be observed until they are normal. At that time another marrow aspirate should be examined to diagnose complete remission or persistent leukemia. No additional induction therapy is necessary if the former pertains, whereas the second induction course should be administered if less than complete marrow remission has been achieved. If residual leukemia without any evidence of maturation in the granulocyte series appears to be present in the first postinduction marrow aspirate, another aspirate should be examined several days later, before reinstituting induction therapy. This is necessary because a marrow recovering from anthracycline drug administration may appear hypocellular and frankly leukemic, but normalize without further therapy. Indeed, an occasional blast may even be found in the blood after anthracycline therapy in a patient who subsequently manifests complete remission without further therapy. In such patients the platelet count may begin to rise, followed within a week by a rise in the granulocyte count. Progressive improvement in both to normal levels as indicated by daily blood counts is usually a harbinger of complete remission. A transient platelet count elevation, often without a concomitant rise in granulocyte count, usually indicates an incomplete response to induction therapy. This must be confirmed by bone marrow examination when the progressive platelet count improvement levels off or reverses. Almost always the marrow examination will confirm an incomplete response in that situation. However, rarely the marrow examination may lead to confusion due to the presence of a megaloblastic maturation arrest in the granulocyte and erythroid series and no evidence of leukemia. In such cases the patient may be folate depleted, especially if significant mucous membrane toxicity occurred during treatment. Such patients may have a dramatic response to daily physiologic doses of parenteral folate, with morphologically normal marrow and normal blood counts evident 10–14 days after initiation of folate therapy. In other cases, all criteria for the diagnosis of complete remission may be present except for the continuation of significant thrombocytopenia and a continuing need for platelet transfusion despite an adequate number of megakaryocytes in the marrow. Most such patients will respond with a normal platelet count to daily, oral low-dose cyclosporin A administration (100–200 mg per day) for unknown reasons [195]. In such patients, cyclosporin A may need to be continued indefinitely. Such patients are designated CRp in many studies [107, 108] and may have impaired long-term prognosis [109]. These guidelines for reassessing the marrow after induction therapy will reduce the number of patients who receive a second induction course needlessly. The common practice of reassessing the marrow at day 14 makes no sense at all [196–198] and has led to overtreatment of many patients.

Many prognostic factors influence induction therapy results. There is an inverse relationship between time to achieve complete remission after one course of chemotherapy, and disease-free and overall survival in patients with AML [199]. The patient's age has the most consistent influence on results. Elderly patients, especially those older than 70 years, are less likely to withstand the severity of treatment [200, 201]. It is not entirely clear why this is so, but other coincidental medical problems such as cardiopulmonary disease common in the elderly may make them less likely to sustain treatment without intolerable toxicity. Poor marrow reserve usually found in the aged may delay or disallow bone marrow recovery after treatment and facilitate infectious complications. In addition, poor-prognosis karyotypes are more frequent in the elderly, whereas good-prognosis karyotypes are more frequent in young adults [200]. For these reasons and others, some have advocated less intensive therapy for elderly patients [202]. Others disagree [203]. Clearly, elderly patients require the maximum in supportive care if intensive treatment is to be given. When intensive treatment is given in a setting of maximum supportive care it is likely to be successful in elderly patients and is likely to yield more benefit than less aggressive therapy without additional significant toxicity [203–205]. In the near future, peripheral blood testing for the absence of minimal residual disease by quantitating the level of molecular mutations that remain after therapy may become the accepted method of documenting response to therapy [198].

Although in many studies remission duration in the elderly treated with standard induction regimens has been poor [200], remission duration and survival have been prolonged in others [205]. Elevated blood urea nitrogen concentration, poor performance status, high peripheral blood blast count, and hepatomegaly have been reported to be particularly poor prognostic factors for survival in the elderly with AML [206]. The treatment of elderly patients is more fully discussed below.

Patients with secondary AML, discussed in Chap. 24, and AML developing after a preleukemic phase or myelodysplastic syndrome [207] have a poorer response rate, response duration, and survival than do patients with de novo AML.

The bone marrow and blood become normal morphologically and the quantities of various cellular elements normalize absolutely and relative to each other in the majority of patients when complete remission is achieved. Some complete responders, however, manifest myelodysplastic changes permanently after stem cell damage from chemotherapy but produce normal blood cells in normal numbers [208, 209]. Remission is usually explained by the premise that chemotherapy kills most of the abnormal cells and allows residual normal stem cells to repopulate the marrow and function normally. However, some evidence suggests that remission may result from maturation and

differentiation of leukemia cells induced by standard chemotherapy [210–212]. Rarely, patients in remission are noted to have Auer rods in otherwise normal granulocytes [212, 213] and some patients have been noted to degranulate mature granulocytes before other evidence of relapse is apparent. In addition, the normal leukocytes of some patients in remission express reverse transcriptase activity characteristic of leukemic cells and uncharacteristic of normal leukocytes [214]. These observations, together with the fact that a large number of agents, including many chemotherapeutic agents, are known to cause differentiation and maturation of leukemic cells in vitro [215, 216], suggest that remission does not necessarily derive from the cytotoxicity of induction therapy alone.

Postremission Therapy

It is universally agreed that postremission therapy prolongs complete remission and enhances the cure rate in AML. From the mid-1960s until recently, myriad maintenance regimens were tested. These regimens were usually given for a finite period each month, were usually less intensive than induction regimens, and usually consisted of multiple drugs most of which were not given during the induction phase of treatment. These treatments may have had a minimal favorable effect on remission duration [217], but were largely unsuccessful. The value of such treatments became even more doubtful when it was demonstrated that the frequency of their administration had no effect on remission duration [136]. Furthermore, some studies during that period suggested that remission duration was not adversely affected by omitting maintenance therapy altogether [218, 219]. Confusion was further compounded by the fact that most studies initiated after 1979 gave better remission duration results than previous studies irrespective of postremission therapy schemes. Most of those studies have employed an intensive induction regimen with an anthracycline and cytosine arabinoside in a schedule and doses similar to the treatment in Table 20.5. Not only have those studies resulted in superior response rates and durations compared with previous ones, but also the number of disease-free long-term survivors resulting from them was significantly greater [136, 138]. These observations suggest that the efficacy of induction chemotherapy is one important determinant of remission duration. This concept is further supported by several idarubicin studies [157, 159]. Such has also proved to be the case in other highly treatable hematologic neoplasms such as advanced Hodgkin's disease. Some biochemical substantiation of that contention has been offered by Rustum and Preisler [220], who found that patients with the longest remission durations were those whose pretreatment leukemic cells best activated cytosine arabinoside to cytosine arabinoside triphosphate and retained the activated compound longest intracellularly.

It has been suggested that postremission schemes as described previously are ineffective not because the concept of postremission therapy is wrong, but because the treatments were less intensive than necessary for optimal results. Therefore, a number of studies employing postremission schemes for a finite period that were at least as intensive as induction therapy were instituted [138, 221–226]. The results of such studies have been very impressive. Median durations of complete remission on the order of 18–24 months have been obtained and 20–45% or more of complete responders so treated have remained disease free for at least 15 years [227] after achieving complete remission. The intensive postremission programs, in general, produce results superior to those obtained with previously employed lower dose postremission therapy.

Four types of successful postremission therapy have emerged from studies conducted over the last 25 years. Consolidation therapy, which is usually given as one or more courses of high-dose cytarabine with or without other agents, has become a standard form of treatment [221, 222, 224, 228]. Such programs appear to be most effective when the dose of cytarabine is 1–3 g/m^2 given every 12 h, and doses of 3 g/m^2 are commonly employed on that schedule in patients under 60 years of age [164] while doses of 1.0–1.5 g/m^2 are used for elderly patients [156]. However, the optimal dose between 400 mg/m^2 and 3 g/m^2 on such schedules remains to be determined. This is important, since cytarabine toxicity escalates steeply above doses of 500 mg/m^2 given twice daily [229]. Treatment with a high-dose consolidation program is outlined as option I in Table 20.6. Other more complicated regimens have been studied [223, 230], but results are similar or inferior to those obtained with the regimen in that table.

High-dose cytarabine-based consolidation programs that employ cytarabine doses of 3 g/m^2 are toxic and associated with a death rate during remission of approximately 10%. Patients with hepatic or renal dysfunction and older patients tolerate this treatment especially poorly. Older patients are especially prone to severe neurotoxicity from this treatment [222, 266]. Whether cytarabine 3 g/m^2 in multiple doses is more effective than lower doses, such as 1 g/m^2, as consolidation therapy is the subject of much debate [267]. Others have employed lower doses of cytarabine without apparent loss of efficacy, although the lower doses have never been prospectively compared with doses of 1 g/m^2 or higher. Neither GM-CSF [141] nor G-CSF [268] has been particularly useful during the consolidation phase of treatment.

A less toxic but effective approach to postremission therapy utilizes conventional doses of cytarabine and 6-thioguanine given on an open-ended schedule until marrow hypoplasia is achieved [138] and is summarized as option II in Table 20.6. This treatment is associated with only

Table 20.6 Postremission therapy options for adult AML patients in first remission [231]

I. High-dose consolidation therapy of short duration
Example: adapted from Mayer et al. [222]
Regimen: Cytarabine, 3 g/m^2, is given as a 3-h infusion every 12 h on days 1, 3, and 5 for a total of six doses per course, beginning within 1 month of complete remission. Courses are repeated every 28–35 days, depending on marrow recovery. A total of four courses are given
Comment: This regimen resulted in a 44% projected disease-free survival at 5 years for patients 60 years of age or less [222]. Results were significantly poorer and toxicity was prohibitive in older patients. Toxicity was significant in patients over the age of 45 years. Therefore, the regimen may only be generally applicable to patients <60 years old. Serious neurotoxicity (usually cerebellar ataxia) occurred in 12% of patients and was permanent in 40% of patients who experienced it. Other serious toxicity included confluent maculopapular rash and desquamation, conjunctivitis, pulmonary fibrosis, and gastrointestinal tract ulceration. Treatment-related death occurred in 5% of patients. Most effective in patients with favorable cytogenetics
II. Intensive recurring regimen given on an open schedule for 3 years
Example: Dutcher et al. [138]
Regimen: Cytarabine, 100 mg/m^2, as an i.v. bolus and oral 6-thioguanine, 100 mg/m^2, are both given every 12 h until severe marrow hypoplasia is achieved. The treatment is given every 3 months for 3 years beginning 1 month after complete remission is established
Comment: Approximately 10 days of treatment is required to achieve marrow hypoplasia with the first several courses, but only 5–7 days of treatment is necessary after 12–18 months. Results are equal in younger and older patients up to age 75. Toxicity is virtually limited to the bone marrow, and only 1% drug-related deaths during remission have been noted in recent years. An observed 20% disease-free survival at 15 years has been reported [227]
III. Allogeneic myeloablative stem cell transplantation
Examples: Young et al. [232], Clift et al. [233], Bortin et al. [234], Zittoun et al. [235], Cassileth et al. [236], Sakamaki et al. [237]
Regimens: The patient's marrow is ablated with high doses of chemotherapy (usually alkylating agents) with or without total-body irradiation. In some studies ablation with irradiation + alkylating agent resulted in superior relapse rate, disease-free survival, and overall survival compared with alkylating agent ablation only [238]. Marrow or peripheral blood stem cells from an HLA-identical sibling or other source are used in this procedure. Long-term results have been reported to be best in patients without ABCG2 overexpression with intermediate- or poor-risk cytogenetics and age $\le$50 years [239, 240]. Long-term results are poor (67% relapse rate) in patients with molecular evidence of minimal residual disease despite morphologic remission at the time of transplant [241]. There is considerable disagreement in the literature as to which AML patients in remission should undergo this procedure [242–248]. Haploidentical donors and matched unrelated donors may give similar results [249, 250]
Comment: The probability of disease-free survival at 5 years has been estimated to be 45–60%, and treatment-related mortality in remission has been reported to be 25–40% in various studies. Results vary inversely with age, and patients over the age of 45 years do less well. For logistical and other reasons, some patients for which this therapy is planned may never receive it [251]
IV. Allogeneic reduced-intensity stem cell transplantation
Examples: McClune et al. [252], Hemmati et al. [253], Ringdén et al. [254], Pagel et al. [255]
Regimens: Less intensive chemotherapy, usually without radiation
Comment: Performed with related or unrelated donors with similar results. Less nonrelapse mortality than with myeloablative conditioning and similar disease-free survival. Three-year overall survival rates of 45% are reported [256]. This procedure is often recommended for elderly patients [257] and other poor-risk patients [240]. There are no studies comparing reduced-intensity stem cell transplantation with chemotherapy alone
V. Cord blood allogeneic stem cell transplantation
Examples: Sanz et al. [258],Verneris et al. [259], Ballen and Lazarus [260, 261]
Regimens: Cyclophosphamide plus TBI ± fludarabine, as an example
Comment: Leukemia-free survival of 40–50% at 2 years has been reported, but nonrelapse mortality has been high and as many as 1/3 of survivors have extensive chronic GVHD. Marrow recovery in the recipient may be delayed.
VI. Autologous bone marrow transplantation
Examples: Gorin et al. [262], Körbling et al. [263], Zittoun et al. [235], Cassileth et al. [236], Czerw et al [264], Mannis et al [265]
Regimens: Preparation of the patient is similar to that for allogeneic transplantation. Marrow must be harvested from the patient after complete remission is achieved and before high-dose postremission chemotherapy
Comment: Long-term disease-free survival comparable to that obtained with allogeneic marrow transplantation has been reported by various authors. Autologous transplantation is much safer than allogeneic transplantation, with only 3–5% treatment-related deaths during remission observed with the former. Autologous marrow transplantation may, therefore, be preferable for older patients. The relative merits of both major types of marrow transplantation and other high-dose cytarabine-based postremission options have been prospectively evaluated by several large cooperative group studies. Most such studies show no difference in overall survival rates. Whatever advantage in DFS is provided by stem cell transplantation is usually offset by toxic deaths

a 1% death in remission rate and can safely be given to patients up to the age of 75 years. Long-term results are excellent, but this approach has never been prospectively compared with a high-dose cytarabine-based regimen.

Allogeneic bone marrow transplantation after marrow-ablative therapy is described as option III in Table 20.6. Best results have been reported in young patients, and most reported series are heavily weighted with such patients. Preparative regimens usually consist of alkylating agents such as busulfan and cyclophosphamide or busulfan and total body irradiation. The latter was more effective than the former with respect to relapse rate, disease-free survival, and overall survival in a prospective, randomized study of patients with AML [238]. With currently available techniques, patients under the age of 60 years, who have an HLA-compatible sibling donor whose blood

lymphocytes do not react with those of the potential recipient in mixed lymphocyte culture, are considered optimal candidates for the procedure. Postremission consolidation therapy with high-dose cytarabine before allogeneic marrow transplantation for AML in first complete remission does not improve outcome compared with proceeding directly to transplantation after recovery from induction therapy [269]. Allogeneic transplants are more successful when the donor is less than 40 years old [270]. Thus, only 20% or less of patients with AML who achieve complete remission can be considered optimal candidates for allogeneic marrow transplantation using a sibling donor at this time, and some of them actually are not transplanted due to logistical and other problems [251].

Allogeneic bone marrow transplantation utilizing an HLA-matched unrelated donor has been studied in patients who do not have a sibling donor. In one study of 161 patients [271] leukemia-free survival at 5 years was 50 ± 12% for patients transplanted during first complete remission, and the relapse rate was 19% after a median posttransplant follow-up of 2.9 years. There was a direct relationship between the duration of leukemia-free survival and the dose of marrow cells infused. These results closely approach those obtained with sibling allografts and represent significant improvement in the efficacy of this procedure.

Many transplant experts think that the future of allogeneic stem cell transplantation lies with cord blood and/or reduced-intensity allogeneic transplantation [272]. Early results are encouraging, but most studies are small, and no studies in which those procedures are compared with chemotherapy alone exist to date.

Relapse after allogeneic stem cell transplantation occurs at about the same rate as in non-transplanted patients. The median time to relapse after transplantation is approximately 7 months, and less than a quarter of relapsed patients survive 1 year [273].

Autologous bone marrow transplantation, option VI in Table 20.6, has been developed more recently than allogeneic transplantation as a postremission option for AML, and in some studies [235, 236, 274], but not all [275, 276], results have been equivalent to those obtained with allogeneic transplantation. Marrow recovery seems to be more rapid after transplantation with allogeneic peripheral blood stem cells compared with marrow cells [277, 278] but there may be a higher incidence of relapse with peripheral blood stem cells [279]. Autologous bone marrow transplantation with [280, 281] and without [235, 275] ex vivo purging appears to yield similar results.

Disease-free and overall survivals for AML patients transplanted with allogeneic stem cells during first remission have been reported by innumerable investigators from around the world. The 5-year disease-free survival rate projected from actuarial analysis in most studies has ranged from 30 to 50%, and relapse rates of 15–25% have been reported. Occasionally, relapses are extramedullary [282], late [283], and possibly due to induction of leukemia in the graft [284, 285]. Most adult long-term survivors of marrow transplantation have been reasonably, if not entirely, well. In one large study, patients with no recurrence of leukemia at 2 years had an overall 82% chance of being alive in complete remission at 9 years following transplantation regardless of the type of transplant. Patients allografted, however, experienced a lower frequency of late relapse than patients autografted [286]. Females had a lower relapse rate than males, an observation also reported after chemotherapy alone [100]. In another large similar study [287] of patients who were free of AML 2 years after allogeneic transplantation, mortality remained higher than in the normal population through the ninth year post-transplantation. Recurrent leukemia was the major cause of death. Haploidentical transplants may give the same results as matched sibling donor transplants [250].

There is no doubt that both allogeneic and autologous bone marrow transplantation can cure AML. The question is the relative frequency of cure from these and other methods. In an effort to answer the question, a number of prospective studies were performed in which allogeneic or autologous bone marrow transplantation and intensive consolidation chemotherapy alone were compared [197, 221, 224, 235, 288–290, 307]. The results have been quite similar in most of these trials. Allogeneic bone marrow transplantation results in fewer leukemic relapses than consolidation chemotherapy alone, but overall survival of the two groups of patients is similar, and results with autologous transplantation are often in the middle [235] with respect to relapse rate.

The high death rate during remission after allogeneic transplantation is primarily due to acute graft-versus-host disease (GvHD). Unfortunately, treatments that reduce the incidence of acute or chronic GvHD, with the possible exception of thalidomide [291], usually lead to an increased incidence of leukemic relapse [292], since the undesired GvHD effect cannot yet be fully separated from the desired graft-versus-leukemia effect of allogeneic transplantation. Some data suggest that patients with favorable cytogenetics have longer disease-free survival after either autologous or allogeneic bone marrow transplantation than with consolidation chemotherapy alone [293, 294] and that patients with unfavorable cytogenetics fare best with allogeneic bone marrow transplantation, but these data need to be confirmed in a larger series. Chronic GVHD can be a serious problem for responders to allogeneic transplantation and its incidence has increased in recent years [295]. Recent data suggest that donor EBV+ status significantly increases the risk of chronic GVHD in the transplant recipient [296], and that low-dose interleukin-2 is highly effective in controlling steroid-refractory GVHD [297]. See Chap. 50 for information on management of the transplant patient.

Treatment of Refractory and Relapsed AML

Although significant improvement has been made in the therapy of AML and the number of potentially cured patients has increased in recent years, most patients still relapse after complete remission and ultimately die of their disease [192]. However, many relapsed patients respond to reinduction therapy with durable remissions and some of them, especially those with favorable cytogenetics such as inv(16), appear to be cured after obtaining a second complete remission. Complete responses to reinduction therapy are much more common in patients who have relapsed after an initial complete response than in patients who were refractory to initial therapy [298], and more common and more durable in patients whose first remission was longer than 1 year. Drug resistance in AML may be partially due to increased glycolysis in resistant AML cells, and this abnormality may be actionable [299].

Patients with relapsed or refractory disease are candidates for further therapy unless serious comorbidities or residual toxicity from prior therapy prevents it. Relapsed patients whose first remission was >1 year are usually retreated with the same induction regimen to which they initially responded. Others are candidates for regimens that they have not previously received followed or not by stem cell transplantation, or stem cell transplantation alone. The longer the first remission and the younger the age of the patient, the better the results with any so-called salvage therapy. Combinations of agents such as mitoxantrone and etoposide are associated with a second complete response in 25% of patients in first relapse and a median overall survival of <8 months [300–302].

A large number of studies in relapsed or refractory disease have used various doses and schedules of cytarabine with and without mitoxantrone [303]. Most yielded a complete response rate between 30% and 50%, depending on patient age and other prognostic factors. No such regimen appears to be superior to another. Although these regimens are popular in various quarters, they leave much to be desired. Relapsed or refractory patients with AML should be seriously considered for clinical trials, since to date there is no standard approach to them.

The combination of fludarabine, cytarabine, and G-CSF has been found to be quite active in poor-prognosis AML. The combination of nucleosides results in enhanced intracellular accumulation of Ara-CTP, the intracellularly active form of cytarabine, and increased DNA damage. G-CSF was thought to enhance the cytotoxicity of cytarabine by recruiting cells into the S-phase of the cell cycle and render them more sensitive to cycle-specific drugs [191, 233], but it does not appear to do this sufficiently to improve clinical results [234]. Results appear to be excellent with this regimen, with or without G-CSF, although it is quite toxic. Opportunistic infections with unusual organisms are rare but severe neurotoxicity [304–306] detracts from its appeal. Furthermore, fludarabine-based induction therapy does not overcome the negative impact of multidrug resistance proteins' overexpression [307].

The addition of gemtuzumab ozogamicin [193] to intensive reinduction therapy may improve those results and allow more patients to survive long enough to receive a stem cell transplant [308]. Recently, high-dose cytarabine, mitoxantrone, all-*trans* retinoic acid, and gemtuzumab ozogamicin was studied in 93 patients aged 18–60 years who were refractory to one cycle of standard induction therapy. The complete response rate was 51% and for patients who responded to this regimen and went on to an allogeneic stem cell transplant the 4-year survival rate was 49% [309]. These results are excellent and deserve confirmation. Others have reported similar results with a fludarabine-containing regimen [310]

Therapeutic results for patients with core-binding factor AML in first relapse are more encouraging. Such patients appear to be more sensitive to gemtuzumab ozogamicin than others, and in one study of 48 patients aged 16–76 years, patients treated with regimens including that agent had a complete second remission rate of 88% and a 5-year overall survival of 51% [311]. Unfortunately, core-binding factor AML patients account for a minority of AML patients.

New agents with major activity against relapsed or refractory AML continue to be identified and recently the specificity of new antileukemic agents has improved greatly. There is renewed interest in azacitidine, which demonstrated significant activity against de novo AML in early phase II studies [312]. Recent studies in patients who relapse after stem cell transplantation [313–316] or elderly patients are encouraging [317] as are data with a similar agent, decitabine [318, 319].

Carboplatin yields complete or partial responses in approximately 30% of relapsed or refractory AML patients [320]. No combinations including this agent have been studied in this setting, however.

Selective ablation of the leukemic clone in AML is theoretically possible with an anti-CD33—calicheamicin immunoconjugate, since CD33 is expressed by most AML cells but not by normal hematopoietic stem cells [321]. Gemtuzumab ozogamicin (GO) was such a conjugate tested clinically. A complete response was reported in 30% of 142 elderly patients with AML in first relapse with that agent [322]. However, more recent studies have been very disappointing. Martin et al. [323] reported that the addition of GO to a fludarabine, cytarabine, and idarubicin regimen failed to improve the outcome of treatment for relapsed or refractory patients. Yamaguchi et al. [324] reported that GO alone had little single-agent activity in relapsed or refractory patients, and Litzow et al. [325] reported a 12% CR rate in a phase II study of cytarabine plus GO in refractory or relapsed patients.

No patients with an initial CR of <6 months or with multiple relapses responded. Löwenberg et al. [326] reported similar results in patients ≥60 years of age in first CR. It should be mentioned that the agent has occasionally been associated with veno-occlusive disease [327]. Balaian and Ball [328] observed that CD33 upon ligation with anti-CD33 downregulates cell growth in a Syk-dependent manner, and demonstrated in vitro a correlation between GO antileukemic activity and Syk expression. Blocking Syk expression rendered AML cells unresponsive to GO, but upregulating Syk by exposing the cells to azacitidine resulted in enhanced GO activity. If these results can be confirmed it would be extremely interesting to study the sequential combination of azacitidine and GO in the clinic.

FLT3 mutations occur in approximately 30% of patients with AML and are associated with shorter disease-free and overall survival after initial or relapse therapy [329] except in patients with acute promyelocytic leukemia. These findings apply especially to younger patients [330]. Pemmaraju et al. [331] reviewed the outcome of 128 patients (22 received first salvage therapy) who received FLT3 inhibitors as part of their treatment. Median overall survival was 3.1 months and 4.2 months for those without and with FLT3 mutations, respectively, and all who had FLT3 mutations achieved CR. Mori et al. [332] demonstrated that inhibition of both FLT3-wt and mutant FLT3 is necessary for maximal antileukemic effect against cells that express both types of FLT3.

Metzelder et al. [333] treated 18 FLT3-ITD + relapsed patients with sorafenib and all had a hematological response. Lee et al. [334] reported a CR of several months' duration in an elderly relapsed male with extensive leukemia cutis. Ravandi et al. [335] reported CR in all 15 FLT3-mutated relapsed patients with a combination of idarubicin, high-dose cytarabine, and sorafenib.

Sunitinib is synergistic with cytarabine against FLT3-ITD + AML cell lines, but not cell lines with FLT-wt [336], and clinical responses have been recorded in mutated FLT3 patients [337], but the agent has not been fully studied in the clinic.

Midostaurin (PK412) is a multi-targeted kinase inhibitor with activity against mutant and wt FLT3 AML. In a study by Stone et al. [338] newly diagnosed patients under the age of 61 were induced with daunorubicin, cytarabine, and midostaurin and consolidated with high-dose cytarabine. A complete response was induced in 74% of FLT3-wt and 92% of mutant FLT3 patients, and 2-year overall survival rates were 62% for the latter and 59% for the former. These positive results led to a placebo-controlled international study (CALGB 10603) of 717 patients with FLT3 mutation. In that study at a median follow-up of 57 months the midostaurin arm reduced the risk of death by 23% [177].

Lestaurtinib (CEP-701), another FLT3 inhibitor in clinical trial, was studied in AML patients in first relapse, but results were disappointing [339]. AC220 is a second-generation FLT3 inhibitor with low nanomolar potency and exceptional kinase selectivity [340] and is currently in clinical trial [341].

FLT3 inhibitors provide us with an entirely new, more specific approach to AML treatment. However, in most studies to date, FLT3-ITD inhibition has been transient and resistance has been noted early. Resistance may be due to inadequate dosing, ligand interference, presence of residual dormant FLT3-ITD+ cells, all of these, or as-yet undiscovered mechanisms. It is too early to discard any of these agents or others not discussed, and all merit further study alone and in combination with chemotherapy and/or other agents such as mTOR inhibitors.

There are few data on post-second remission therapy that suggest that one treatment is superior to another. Robles et al. [342] demonstrated that low-dose cytarabine, 10 mg/m^2 given subcutaneously every 12 h until relapse, may have resulted in a second remission duration longer than that expected from no maintenance therapy. If these data can be confirmed, postremission cytarabine will be the first agent demonstrated to prolong second remission. A surprising finding in that study was the fact that 18% of patients in the control group also had second remissions longer than the first [343], which suggests that reinduction therapy was a more effective antileukemic therapy than initial induction and postremission therapy in that group. Therefore, it is essential to perform prospectively controlled post-second remission studies if new active therapies are to be identified, rather than simply to determine "inversion" rates from uncontrolled studies.

Farnesyl transferase inhibitors such as tipifarnib and lonafarnib have entered clinical trial in AML, but to date, results have been disappointing [344–346]. The combination of simvastatin and tipifarnib may be more active than tipifarnib alone against CD34+ AML cells [347], but clinical trials have not yet been done.

A regimen of fludarabine, cytarabine, and liposomal daunorubicin was successful in refractory and relapsed patients and produced a 44% and 56% complete response rate in them, respectively [348]. Remissions were relatively short, and a more recent study demonstrated little or no activity for liposomal daunorubicin in similar patients [224].

There is no doubt that bone marrow transplantation can cure some patients with relapsed and/or refractory AML [349–352]. However, it has been suggested by some that a bone marrow-preparative regimen without stem cell support may yield results equivalent to those reported after transplantation [353]. In a study by Forman et al. [349], 12 adults with AML who failed induction therapy were treated with an allogeneic transplant from a matched sibling and 75% achieved a complete remission. One-third of the complete responders relapsed and two-thirds (ages 20–29) remained in continuous complete remission for approximately 18–108 months. In a larger Seattle study [350] that included some

older patients, allogeneic transplantation was tested in AML patients in untreated first relapse and the 5-year disease-free survival was projected to be 23%. Only one of ten patients with AML given an allogeneic transplant in second complete remission in another study [351] survived in long-term complete remission. Ipilimumab, an immune checkpoint blockade monoclonal antibody, may restore antitumor activity through a graft-versus-tumor effect in patients who relapse after allogeneic transplantation and by this mechanism restore response in such patients. The agent is under clinical investigation [354].

Autologous bone marrow transplantation for patients in first relapse or in second complete remission was studied by Petersen et al. [352]. In all patients, marrow was harvested and cryopreserved during first complete remission. The actuarial probabilities of relapse-free survival at 2 years for patients transplanted in first relapse (21 patients) or second remission (26 patients) were 45% and 32%, respectively. The outcome in patients who were in first relapse was comparable to that of other studies in which remission was induced prior to transplantation, which suggests that there is no clinical disadvantage in proceeding directly to transplantation upon the diagnosis of relapse. Early data from the same institution suggested that the addition of IL-2 to the management of autologous transplant patients in first relapse or later stages may improve outcome [355]. However, recent data suggest no benefit for IL-2 for patients in first remission after autologous marrow transplantation [356, 357].

Some data suggest that autologous stem cell transplantation may actually be more effective than allogeneic transplantation in relapsed or refractory patients [358].

Infusion of lymphocytes from the original marrow donor is highly effective in treating chronic myelocytic leukemia patients who relapse after allogeneic marrow transplantation, but donor lymphocyte infusions are less effective in treating posttransplant relapsed patients with AML [359–361]. The major problem with this form of treatment is the frequent induction of serious GvHD, which can be fatal [362]. However, such infusions can be successful, even for relapse after unrelated donor marrow transplantation [359]. Porter et al. [363] treated 23 patients with AML who relapsed after an unrelated donor marrow transplantation and 42% obtained a complete response with an estimated 1-year disease-free survival rate of 23%. Donor lymphocyte infusions may also be able to eradicate persistent disease after allogeneic hematopoietic cell transplantation [364].

Elderly Patients

Röllig et al. [365] devised a prognostic model for elderly patients with newly diagnosed AML in which karyotype, age, NPM1 mutation status, WBC, LDH, and CD34 expression were of independent prognostic significance for overall survival. This model may be useful in other studies and may be used to stratify patients in future prospective trials. Older patients with CEBPA double mutation, NPM1 mutation, and FLT3-wild type may have significantly better survival after intensive treatment than others [366].

There is no standard treatment for relapsed or newly diagnosed elderly patients (>70 years of age) with AML. As a general rule, the same intensity of treatment used for younger patients should be considered for elderly patients because results tend to be better than with less aggressive therapy for induction. However, elderly patients do not tolerate consolidation with cytarabine 3 g/m^2 and doses half that large are commonly used. As in younger patients, for patients whose initial remission was >1 year, the same induction therapy used initially will likely yield the best results for reinduction. It should be noted that many patients with late relapse (>5 years after CR) relapse with different cytogenetics than they originally displayed [367, 368] and have a poor likelihood of long-term survival after relapse. All elderly patients should be seriously considered for a clinical trial. Some data suggest that elderly patients are less sensitive to anthracyclines than younger patients [369] and resveratrol may be helpful in overcoming this relative resistance [370].

A common approach to induction therapy of elderly patients is low-dose cytarabine (20 mg once or twice a day) by subcutaneous injection for 10 consecutive days every 4–6 weeks. Although the complete response rate with this regimen is <10%, survival is better than with no treatment other than supportive care [371]. Results with new drugs may make this approach completely obsolete. Low-dose cytarabine was studied with volasertib, a selective inhibitor of polo-like kinases, and compared with low-dose cytarabine alone in a randomized study. The combination was given to 87 patients with a median age of 75 years and the complete response rate (31%) and event-free survival rate were double that obtained with low-dose cytarabine alone. Cytogenetics did not influence response. Volasertib added to the marrow and gastrointestinal toxicity of treatment, but did not lead to more deaths.

Clofarabine, a deoxyadenosine analog, was evaluated in elderly patients with AML with good results in previously untreated patients. A complete response was obtained by Kantarjian et al. [372] in 46% of 112 patients aged 60–88 years with a median duration of response >1 year. In a study by Burnett et al. [373] of 106 elderly patients a similar response rate was observed. In both studies the drug was reasonably well tolerated. Kadia et al. [374] studied an induction regimen of clofarabine and low-dose cytarabine alternating with decitabine in 118 patients aged 60–81 years followed by consolidation and maintenance therapy with the same drugs. The complete response rate was 60% overall and 50% in those with adverse cytogenetics. Overall median survival in

the complete responders was 18.5 months. They concluded that the treatment was well tolerated and highly effective in older patients with AML. Takahashi et al. [375] compared clofarabine alone to idarubicin plus cytarabine in elderly patients and found equivalent responses and survival with the two treatments, but less toxicity with clofarabine alone. In a recent study 84 patients aged 40–75 years were treated with clofarabine and cytarabine and 67% of them went on to an allogeneic stem cell transplant that was preceded by a clofarabine-based conditioning regimen. Complete remission was achieved in 60% of patients after transplantation and the 2-year disease-free survival rate was 52%. These results are encouraging, but this was a single-arm study [376] and it is unclear whether these results are better than those that would have been obtained with other treatments. The drug needs further evaluation in combination with others.

Vosaroxin is a first-in-class quinolone derivative that acts as a topoisomerase II inhibitor and does not have the cardiac toxicity of topoisomerase II inhibitors. Its toxicity is primarily hematologic. In a phase II study of 116 previously untreated patients aged 60 years or more, several doses and schedules were studied and in the best group a 35% complete response rate was obtained, with a 1-year overall survival of 38% [377]. In another study vosaroxin was found to give results no better than low-dose cytarabine [378]. Despite other trials showing some activity for this agent [379], it seems unlikely that it will have a role in the treatment of AML.

Decitabine, alone and with other agents [381–383], is currently under evaluation for the treatment of elderly patients with AML but results are too early to fully evaluate [380, 381] or disappointing [382].

Another hypomethylating agent, azacitidine, appears to be somewhat more promising in elderly patients with AML. In one study [383] azacitidine or decitabine alone yielded essentially similar results as intensive chemotherapy in elderly patients. Another study demonstrated that hematologic improvement with azacitidine short of complete response led to improved survival [384]. In a study of 149 previously untreated patients with AML and a median age of 74, including 51 patients with de novo AML considered ineligible for intensive chemotherapy, the complete response rate with azacitidine alone was 33% and the median overall survival was 9.4 months. The 2-year overall survival was 51% in responders in this single-arm study [385]. In a study of low-dose subcutaneously administered azacitidine as maintenance therapy in elderly patients in first remission after standard induction therapy [386] the median overall survival was 20.4 months. The treatment was well tolerated and seems to have been effective. Obviously further study is needed before that impression can be confirmed.

Dombret et al. [387] performed a study of 488 elderly newly diagnosed patients randomized to receive azacitidine or several conventional treatments. Azacitidine was associated with a 3.8-month improvement in median overall survival and was well tolerated. The 1-year survival rate with azacitidine was 46.5%, compared with 34.2% for the other patients. This study provides the greatest impetus for the further study of azacitidine in elderly patients with AML. Unfortunately, to date, studies of combinations of azacitidine with intensive therapy [388] or a histone deacetylase inhibitor [389] are disappointing. On the other hand, azacitidine plus sorafenib may be effective for elderly patients with FLT3-ITD mutations [390], and in patients with FLT3-ITD and NPM1 mutation, azacitidine combined with lenalidomide may be promising postremission therapy [391]. Further studies of azacitidine in AML should take into account that low miR-29c expression by leukemic cells correlates with response to azacitidine by elderly patients [392].

In an early study of the addition of gemtuzumab ozogamicin to standard induction therapy for elderly patients the combination was found to only add toxicity without any benefit [393]. However, as a single agent gemtuzumab ozogamicin significantly improved overall survival compared with best supportive care in patients over the age of 60 years [394]. The drug remains a treatment option for elderly patients who are not candidates for more aggressive treatment if it were available.

The addition of sorafenib to standard induction therapy did not improve outcome in elderly patients, even those with FLT3-ITD mutation [395], although it does so in younger patients with that mutation [396].

A very interesting compound under investigation in elderly patients with secondary AML is CPX-351, a liposomal preparation of daunorubicin and cytarabine in a 1:5 molar ratio. In a randomized study of 309 patients aged 60–75 years comparing that agent to standard administration of daunorubicin and cytarabine in a standard schedule with standard doses, the new agent proved superior in terms of overall survival, response rate, and 60-day mortality [397]. This compound is likely to become widely used for elderly patients with AML.

At least 60% of patients with AML are 65 years of age or older. They are more likely to have an antecedent hematologic disorder, unfavorable cytogenetics, and poorer performance status at diagnosis mainly due to assorted comorbidities. Many, if not most, are judged by their physician to not be a candidate for intensive chemotherapy. Those that are treated have a significantly improved overall survival [398]. On the other hand older patients have a lower complete response rate to standard induction regimens than do younger patients and remission duration is usually shorter as well. Relapsed elderly patients rarely have useful responses to reinduction therapy although this is not always the case. Newer treatments briefly described above have not had a major impact on the results of treatment in elderly patients.

Therefore, treatment of elderly patients remains a major challenge. Hopefully, some newer agents and concepts just entering clinical trial will improve results for them. In the meantime, all elderly patients judged not eligible for standard treatment should be considered for clinical trials.

It should be noted that older patients who achieve remission after intensive or other therapy achieve significant improvements in quality of life, fatigue, and physical function as do younger patients [399].

Central Nervous System Leukemia

The diagnosis and treatment of CNS leukemia are discussed earlier in this chapter. CNS leukemia is an uncommon type of presentation or relapse in adults with AML. The incidence of CNS leukemia has decreased in AML patients since the common usage of infusional cytarabine in induction regimens due to the attainment of therapeutic levels of cytarabine in the CSF with such induction therapy. Nevertheless, about 1–2% of patients who relapse will have CNS leukemia with or without marrow evidence of relapse. These are usually young patients. Intracerebral leukemia is much less common in AML than in ALL, and virtually all adults with AML with CNS leukemia demonstrate meningeal leukemia, or cranial nerve palsy or both. Irradiation of the course of an involved cranial nerve will preserve function of that nerve if done early. Cranial nerve palsy does not respond to intrathecal chemotherapy. Patients with meningeal leukemia with cerebrospinal fluid pleocytosis usually respond to intrathecal chemotherapy, usually cytarabine, and usually do not require cranial irradiation. For reasons that are not understood, patients with AML usually have longer remissions of CNS leukemia after treatment than do patients with ALL [400].

As is the case in ALL, rapid attainment of remission of meningeal leukemia, long duration of initial marrow remission, and absence of cranial nerve palsy are favorable factors for CNS leukemia remission duration after treatment.

Mixed-Phenotype Acute Leukemia

This is an uncommon form of acute leukemia accounting for perhaps 2–3% of all pediatric and adult acute leukemias. When the leukemic blast cells display cytochemical and/or immunophenotypic features of both myeloid and lymphoid blasts the leukemia is referred to as biphenotypic and when there are two populations of cells, one clearly myeloid and the other clearly lymphoid, the leukemia is referred to as bilineal. There are few data to guide treatment for these leukemias but, in general, they are treated with ALL treatment programs and therefore will not be considered further here. For a full discussion of this “entity,” see Wolach and Stone [401].

Future Directions

There is considerable interest in developing molecular tests for the diagnosis of AML [402] and for the detection of minimal residual disease in patients who have achieved complete hematologic remission [403, 404]. Determining the presence or absence of minimal residual disease will allow for informed decisions about whether or not patients require further therapy to potentially achieve cure [405].

New drugs and new techniques are under investigation for the treatment of AML and some may improve therapeutics in the future. Arsenic trioxide and all-trans retinoic acid have been found to induce apoptosis in NPM1-mutated AML cells in the laboratory [406]. Whether this induction can be demonstrated in patients remains to be seen. An old drug, pyrimethamine, was found to have significant activity against human AML in two mice xenograft models [407]. This is an oral agent that could be tested in elderly patients not fit for intensive treatment. A dendritic cell vaccine is being developed for the potential elimination of minimal residual disease and may soon come to clinical trial [408]. Vaccination with polyvalent WT1 peptides in patients with WT1+ AML in remission has been carried out at several institutions and found to be safe and possibly effective [409]. Larger clinical trials will be required to fully evaluate the effectiveness of this promising approach. Blocking MNK kinase activity in an AML xenograft model with merestinib, an orally administered multikinase inhibitor, suppresses primitive leukemic progenitors from patients with AML [410]. Preclinical studies with the agent will continue and ultimately it may come to clinical trial. Another approach under investigation involves targeting miR-126 in leukemic stem cells with antagomiR-126 nanoparticles [411]. Higher expression of miR-126, a marker for leukemic stem cells, is associated with a poor prognosis in older patients with AML and a normal karyotype treated with conventional treatment. A similar approach has been demonstrated to be feasible against FLT3-ITD+ AML [412]. Although interesting, this concept is not yet ready for clinical exploration. Another novel concept, inhibition of certain mitochondrial proteases as a leukemia therapy, is under laboratory investigation and likely to undergo clinical study in the future [413]. In an intriguing study reported in abstract only to date, Hazenberg et al. [414] reported that AML patients cured after allogeneic stem cell transplant produce tumor-specific cytotoxic antibodies that kill AML blast cells in vitro and in mouse models. Perhaps such antibodies can be used to treat other AML patients. A trial in elderly patients would be of great interest.

New prognostic markers for the disease that may serve as therapeutic targets continue to be identified. CD11b expression in a meta-analysis of 2619 patients was shown to be associated with a poorer outcome in patients with AML [415]. CTNNA1 hypermethylation, found in 25% of patients with AML, is an independent predictor for poor relapse-free survival [416].

Recent data have confirmed that smoking may influence the onset and pathogenesis of AML [417]. Hopefully these observations will be more fully explored.

Survivors of AML have an incidence of oral and pharyngeal cancer significantly higher than that of the general population for unknown reasons [418]. Is there a common viral etiology to both diseases, such as HPV?

There is a high risk of hepatitis B reactivation among patients with acute myeloid leukemia and prophylaxis with anti-HVB vaccine has been recommended [419]. It should be noted that decades ago several studies indicated that chronic viral hepatitis had a *favorable* effect on AML prognosis [420–422]. Perhaps the relationship between viral hepatitis and AML should be reexamined.

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21 Acute Promyelocytic Leukemia

Peter H. Wiernik, Robert E. Gallagher, and Martin S. Tallman

Introduction

Acute promyelocytic leukemia (APL) is designated M3 in the French-American-British (FAB) classification. Because of its unique clinical features and unique response to certain differentiation inducing agents, and because of our advanced understanding of the molecular biology of this leukemia, APL deserves to be presented and discussed in detail, apart from the other acute myeloid leukemias.

APL was first described by Hillestad in 1957 [1, 2]. Three patients were described with the characteristic morphology of hypergranular APL, hypofibrinogenemia, and a hemorrhagic diathesis [1]. Caen et al. [3] established that the hemorrhagic syndrome was directly related to the proliferation of the leukemic cells in APL, and Bernard et al. [4] provided a precise description of the disease in a presentation that included 20 patients. Bernard et al. [5] subsequently discovered the unusual sensitivity of this AML variant to daunorubicin, an important observation revisited in the all-trans retinoic acid (ATRA) era discussed in detail next. Rowley et al. in 1976–1977 described the balanced cytogenetic translocation (15;17) in APL [6, 7] and found it to be present in virtually every patient (Fig. 21.1) [8]. Kantarjian et al. [9] reported that chemotherapy could induce complete remissions (CR) in APL without inducing marrow hypoplasia and that remission was often the result of a gradual morphologic evolution, an observation later confirmed by Stone et al. [10] who suggested that the mechanism of remission in APL may be leukemic cell differentiation. Breitman et al. [11] demonstrated that maturation and differentiation of leukemic cells thought to be human APL cells (HL-60 cell line) could be accomplished in vitro by several agents including retinoids. Huang et al. [12] reported the first large series of APL patients treated with oral ATRA and demonstrated a phenomenally high rate of relatively brief CR. Fenaux and colleagues [13] treated APL with ATRA and standard chemotherapy and reported results superior to those obtained with either treatment alone, and subsequently, arsenic trioxide was identified as the most active single agent in APL [14]. Coincident with these treatment advances, there has been an explosion of knowledge of APL at the molecular level. All of these events taken together have contributed to the fact that APL is now the most curable variant of acute myeloid leukemia in adults [15, 16]. For a more detailed history of APL, the reader is referred to Bernard [17].

Molecular Biological Aspects of APL

Overall Perspective

Perhaps more is known about the molecular biology of APL, both in terms of genetic mechanism and potential for tumor cell specific therapy, than for any other specific type of human cancer. The historical background underlying this statement derives from two distinct lines of investigation. One is genetic in nature, beginning with the discovery in 1977 that the APL phenotype of AML is consistently associated with a reciprocal translocation between chromosomes 15 and 17 [7]. The second is biological, stemming from the finding in 1981 that APL cells are unique in their property of undergoing terminal differentiation after exposure in short-term tissue culture to supraphysiological concentrations of the naturally occurring metabolite of vitamin A, all-*trans* retinoic acid (ATRA) [18]. The clinical relevance of the

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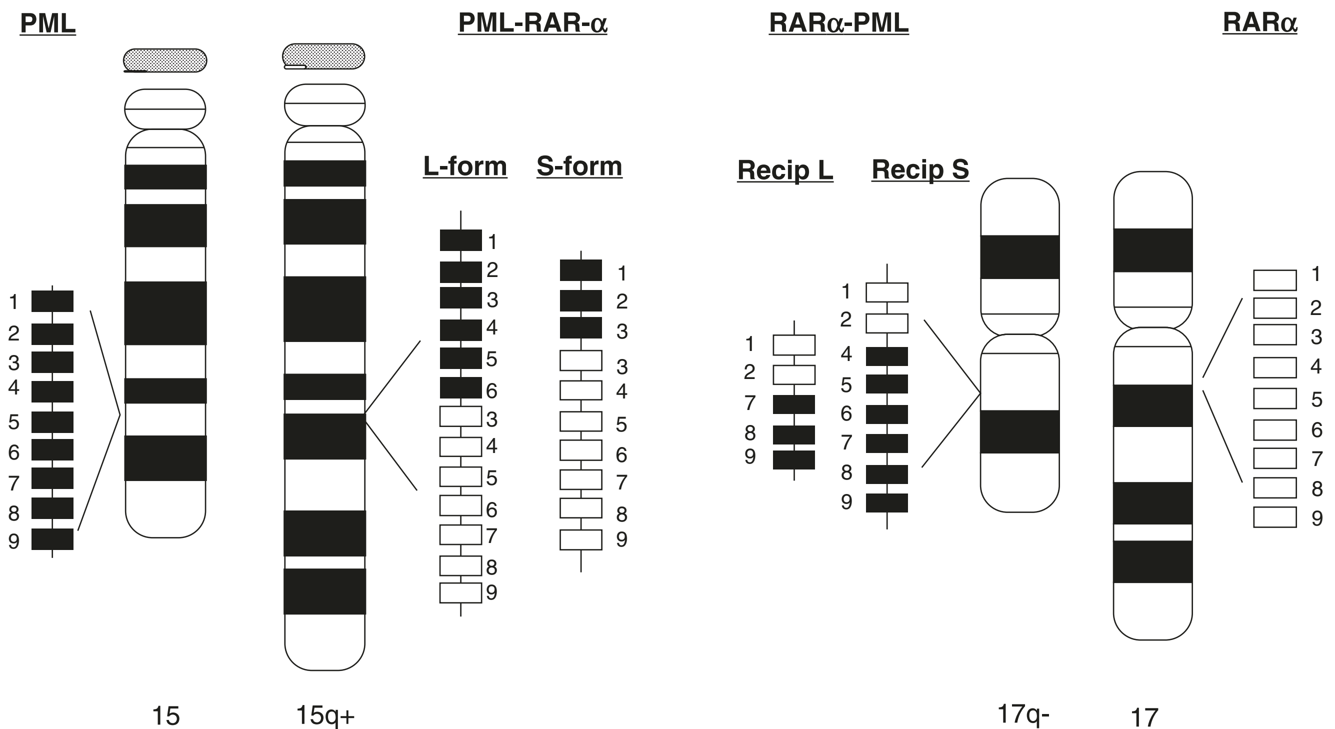


Fig. 21.1 Normal and translocated chromosomes 15 and 17 and the corresponding mRNA transcripts for the normal and hybrid forms of PML and RARα present in a typical case of APL. The vertical chains of *boxes* represent the 9 exons of both the PML (*filled boxes*) and RARα (*empty boxes*) gene transcripts. The short (S) and long (L) forms of PML-RARα result from break sites in PML introns 3 and 6, respectively, whereas RARa is uniformly broken in intron 2

in vitro findings was demonstrated in 1988 when Chinese investigators reported that ATRA produces complete remissions in a high percentage of APL patients [19]. In 1990, there was a remarkable confluence of these two investigative lines with the discovery that the t(15;17) consistently produced breakage of the retinoic acid receptor-alpha (RARα) gene on chromosome band 17q11–21 [20–22]. This seminal discovery provided instant access to a wealth of molecular information that had been developed related to the RARα gene since its discovery in 1987 [23, 24], which, as a ligand-dependent nuclear transcription factor that mediates cellular responses to ATRA, had obvious implications for the selective action of ATRA in APL cells. The following year, the fusion partner of the RARα gene from chromosome 15 was identified and was originally called myl but subsequently renamed PML (for ProMyeLocytes or ProMyelocytic Leukemia) [25–27]. Thus, the two hybrid gene products which result from the reciprocal t(15;17) in APL are PML-RARα and RARα-PML (Fig. 21.1).

In the intervening years, six alternative fusion gene partners of RARα have been discovered in rare cases of APL, each associated with a unique chromosome translocation (le 23.1; see discussion that follows) [28–33]. All of the fusion proteins include the same amino-truncated portion of RARα, indicating the central role of RARα in the pathogenesis of APL. The involvement of the PML gene in >98% of APL cases implies that it also contributes some essential function to pathogenesis, which has gained support from a variety of findings. Although the alternative fusion genes, generically referred to as X-RARαs, are rare and, hence are of limited clinical impact, they have provided useful information about the molecular pathogenesis of APL and about the effect of therapeutic agents on APL cells [34, 35].

Another milestone in the history of APL were mid-1990s Chinese reports that arsenic trioxide (ATO) was, like ATRA, selectively effective for the clinical treatment of APL but that the molecular biological response was different [14, 36]. These differences are presented in some detail in the succeeding sections as they relate both to the initial APL response to ATRA and ATO and to the development of resistance in the declining fraction of patients who experience disease relapse after treatment with these highly effective agents.

Studies of the molecular biology of APL have been markedly abetted by the establishment of the fusion gene-positive APL cell line NB4 in 1991 [37]. The derivation of this cell line importantly permitted more specific molecular evaluations than possible with the antecedent ATRA-sensitive prototype cell line HL-60 that had been isolated from a patient with APL features but lacked the PML-RARα fusion gene [38, 39]. Additionally,

the development of transgenic mice (TM) bearing the fusion genes has provided an important resource for evaluating the role of the different fusion genes in APL pathogenesis and treatment [35, 40]. Recently, the TM models as well as studies with fusion gene-transduced hematopoietic progenitor cells have provided important insights into attributes of the so-called APL leukemia-initiating cell (LIC), which have important implications for treatment strategies [41–43]. However, detailed biological and molecular characterization of these LIC that are required for sustaining and propagating the disease must await their physical separation for analysis.

RARα and the Essentials of Nuclear Receptor Function

RARα is a member of the steroid/thyroid hormone receptor gene superfamily, which encodes proteins that function as ligand-dependent regulators of gene transcription [44]. Most essentially, these proteins contain two domains, a DNA binding domain (DBD) near the amino terminus and a ligand binding domain (LBD) near the carboxy terminus (Fig. 21.2). The DBD contains two characteristic zinc finger motifs that bind the receptors to specific oligonucleotide sequences, hormone response elements (HREs), in the promoter region of select genes, many of which have central effects on cell and tissue growth, differentiation, and homeostasis. RARs, which include separate genes for RARβ and RARγ in addition to RARα, belong to one major branch of the steroid/hormone receptor superfamily, along with the thyroid hormone receptors and vitamin D3 receptor. These nuclear receptors have the common property of residing in the nucleus in a bound state to their respective HREs, consisting of two direct repeats of the hexanucleotide (A/G)G(G/T) TCA (Figs. 21.2 and 21.3a). These receptors also share the property of binding to HREs as a heterodimer with common adapter proteins called retinoid X receptors (Figs. 21.2 and 21.3a), of which there are also three different genes (RXR-α, β, and γ). The discriminator for HRE specificity is the number of nucleotides between the two direct repeats, which is 2 or 5 for retinoic acid response elements (RAREs), 3 for vitamin D3 RE (VDRE), and 4 for thyroid hormone RE (TREs). Additionally, HRE spacers with one nucleotide have specificity for RXR homodimers or, in some cases, may heterodimerize with RAR in reverse polarity [45]. Through expression of different combinations of RXR/RAR heterodimers, variations in RAREs, competition between nuclear receptors and alternative transcription factors for limiting quantities of RXRs, and differences in retinoid ligand utilization, the retinoid receptor system can generate enormous heterogeneity which has been related to the discriminatory, instructive

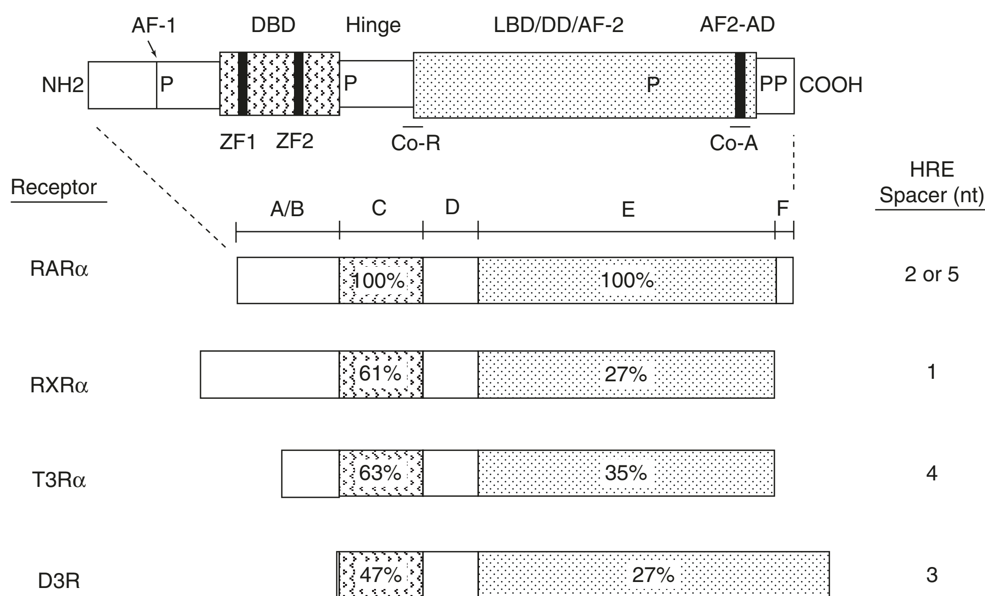
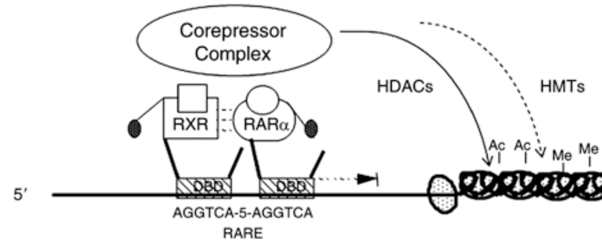
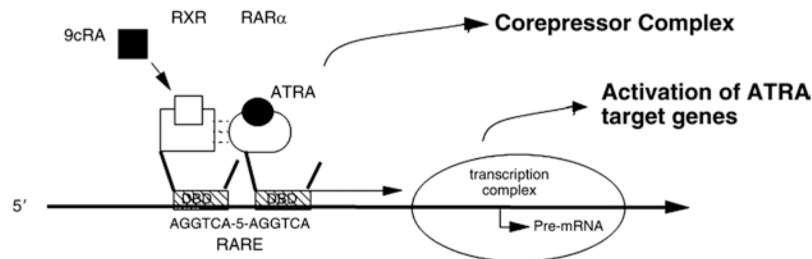


Fig. 21.2 Structure of RARα and its homology to related members of the RAR-RXR-T3R-D3R branch of the steroid-thyroid hormone receptor gene superfamily. AF1, activator function 1 (ligand-independent) domain; DBD, DNA-binding domain; LBD/DD/AF2, shared ligand binding, heterodimerization (RXR) and activator function 2 (ligand-dependent) domains; ZF1 and ZF2, zinc fingers 1 and 2; AF2-AD, AF2 activation core domain containing consensus sequence for binding coactivators (Co-A); CoR, corepressor-binding domain; P, phosphorylation sites. A through F are standard assigned regions/domains of these proteins. Vertical *arrow* indicates the universal break site in RARa between the A and B domains that occur in formation of the various fusion proteins. Percentage numbers indicate the degree of amino acid sequence homology of the DNA-binding (C-regions) and ligand-binding (E-regions) domains of each receptor protein to RARa. HRE, hormone response element; the numbers beneath indicate the number of nucleotides (nt) between the two one-half site direct repeats, PuG(G/T)TCA

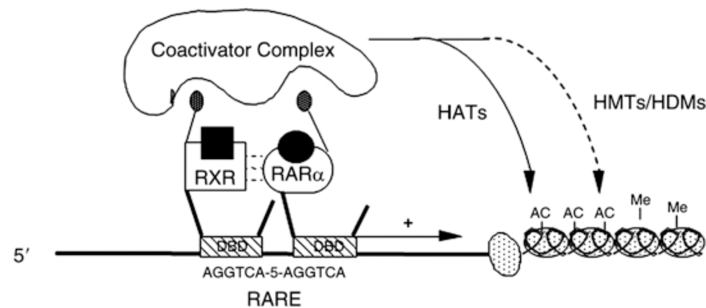
a Co-repressor complex inhibits transcription by RXR/RARα heterodimer



b Transcriptional activation by ligant-bound RXR/RARα heterodimer



c Coactivator complex



d Multi-component transcription activation complexes

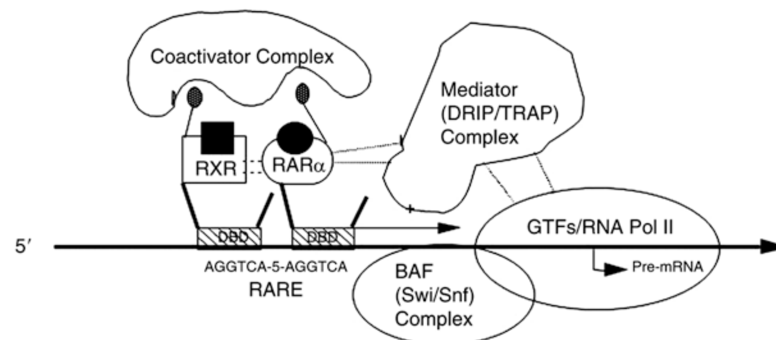


Fig. 21.3 Model for regulation of an ATRA-responsive gene promoter. (**a**) Native configuration of a RXR/RARα heterodimer bound to a canonical genomic retinoic acid response element (RARE) in the absence of ligand (ATRA). The carboxy-tail of the receptors containing the last of 12 helical motifs (*hatched oval*) is in an open position, and the receptors are engaged by a complex of corepressor proteins. A component of this complex is a histone deacetylase (HDAC) enzyme which removes acetyl (Ac) residues from the tails of select histone lysines, favoring a compact, repressed chromatin state that impedes gene transcription (*dotted arrow*). Histone methyl transferases (HMTs) directed to specific lysine residues can also contribute to the repressive effect. (**b**, **c**) Binding of ATRA to the RARα component of the heterodimer, Fig. 21.3 (continued), results in tightening of the receptor, including closure of the 12th helix over the bound ligand, associated with expulsion of the corepressor complex and recruitment of the alternative coactivator complex. This change also produces changes in the heterodimer such that the RXR component becomes permissive for binding its specific ligand 9-cis retinoic acid. The coactivator complex is nucleated by a p160/SRC family member and recruits other proteins, such as CREB-binding protein (CBP/300), with histone acetyltransferase (HAT) activity. Increased histone acetylation loosens chromatin structure, allowing access of transcription factors to promoter sites and fostering transcriptional activity. Highly site- and context-dependent methylation changes executed by specific HMTs and histone demethylases (HDMs) importantly modulate chromatin regulation of transcription. (**d**) Two additional multiprotein complexes complement or succeed the p160 coactivator complex to enhance access and functional activity of general transcription factors (GTFs) to transcribe RNA from active promoters, the mediator (DRIP/TRAP) and BAF (Swi/Snf-homologous) complexes (see text)

role of retinoids in different tissues and cells types during development [44].

In addition to the DBD and LBD domains, respectively, designated as the C and E regions, there are three other subregions of RARα (Fig. 21.2). The A/B-region has ligand-independent transcriptional activation function (AF-1). The D-region serves a rotational or "hinge" function related to heterodimer formation on RAREs. The function of the F-region, which is unique to RARs, is unknown. The E-region of RARα has been analyzed by many different methods, including site-directed mutagenesis and crystallographic analysis in the presence and absence of ligand [44, 46]. Such analysis has defined the three-dimensional structure of the region to consist of 12 α-helices and 2 β-strands linked by a series of loops. From this conformational model, it has been determined that key amino acids from various components of the LBD contribute to the formation of a binding pocket for ATRA, while those from a more restricted area stabilize a RXR/RAR dimerization interface through noncovalent bond interactions. In the absence of ligand, the apo-receptor forms a corepressor complex on RAREs in gene promoter regions, which impedes transcription of the associated gene (Fig. 21.3a). Central to this complex is the corepressor protein (SMRT or N-CoR) which directly interacts with each component of the apo-receptor and which recruits other proteins with histone deacetylase (HDAC) enzyme activity [47]. By removing negatively charged acetyl groups from lysine residues in the tails of histone proteins, HDACs increase the condensation of DNA-associated nucleosomal chromatin and inhibit gene transcriptional activity. The formation of the holoreceptor by entry of ATRA into the binding pocket produces tightening of the receptor, a prominent feature of which is a closing of the 12th, carboxy-terminal α-helix over the opening to the occupied binding site (Fig. 21.3a, b). This configurational shift results in displacement of the corepressor complex and recruitment of the coactivator complex (Fig. 21.3b, c). The core component of this complex is a p160 coactivator protein that recruits proteins with histone acetyl transferase (HAT) activity. By restoring acetyl groups on histone lysine residues in nucleosomes, these enzymes foster decondensation of chromatin and increased gene transcription. Thus, the essence of RARα function is as a sensitive switch to either repress or activate transcription from RARE-containing gene promoters in the absence or presence of ATRA, respectively.

The previously simply presented process is, in fact, highly complex and dynamic involving cell and gene promoter context-variable modifications of a multitude of molecular components that modulate transcriptional activity. At least eight classes of protein modifications have been identified that can affect the interactions and activities of these components [48]. Acetylation of histones, as described earlier, is one crucial representation of such modification. However, there is increasing documentation of the regulatory role of lysine acetylation of many nonhistone proteins, including RARα [49, 50]. Related to retinoid-mediated transcription, it has been proposed that acetylation of a p160 coactivator protein that results in disassociation of the coactivator complex from a nuclear hormone (estrogen) holoreceptor is likely commonly applicable to nuclear receptors and that this dissociation is required for engagement of the Mediator or DRIP/TRAP complex [51, 52]. The latter and another multicomponent complex, the ATP-dependent "chromatin remodeling machine" called SWI/SNF (yeast) or BAF (man), produces further chromatin decondensation essential for final engagement of the basal transcription apparatus and synthesis of mRNA by RNA polymerase II (Fig. 21.3d) [52–54]. Methylation of lysine and arginine residues in histone proteins in concert with methylation of DNA is considered to have an overall repressive effect on transcription. However, histone methylase enzymes have higher specificity than acetylases, and there is marked heterogeneity of effect depending on cellular context and the position and level of methylation (mono-, di-, or trimethylation). A defined set of histone lysine residues have been identified for which methylation or acetylation act reciprocally and in some instances in opposition to the more general effects of these protein modifications. These antagonizing "histone marks" for transcriptional repression or activation, referred to loosely as the "histone code," provide a reading guide for the chromatin modifiers involved in mediating the transcription process [55]. Protein modification by phosphorylation plays a major role in modulating retinoid-mediated transcription at all levels of the process [52]. There are at least four phosphorylation sites in RARα (Fig. 21.1), which are targeted by several different kinases, including the signaling kinases MSK1, PKA, and PKC. These and additional kinases also target RARα-associated cofactors and components of the intermediary and basal transcription complexes, most often with a stimulatory effect on transcription (but with many particular variations) [52].

PML and PML Nuclear Bodies

Structurally, PML is characterized by a conserved "tripartite motif" at the amino-terminus, which consists of a cysteine/histidine-rich (Cys3HisCys4) cluster called the RING domain, followed by two alternative cysteine/histidine clusters called B-boxes, succeeded by an α-helical coiled-coil domain (Fig. 21.4b) [56]. This RBCC structure is shared by eight other members of a large gene family, two of which in addition to PML can form oncogenic hybrid proteins as a result of tumor-associated chromosome translocations. Although all three of the cysteine/histidine clusters bind to zinc ions, which are characteristic of DNA-interacting zinc finger motifs, there is no evidence that PML directly interacts with DNA. Rather, the RING domain functions through protein–protein interactions facilitated by hydrophobic

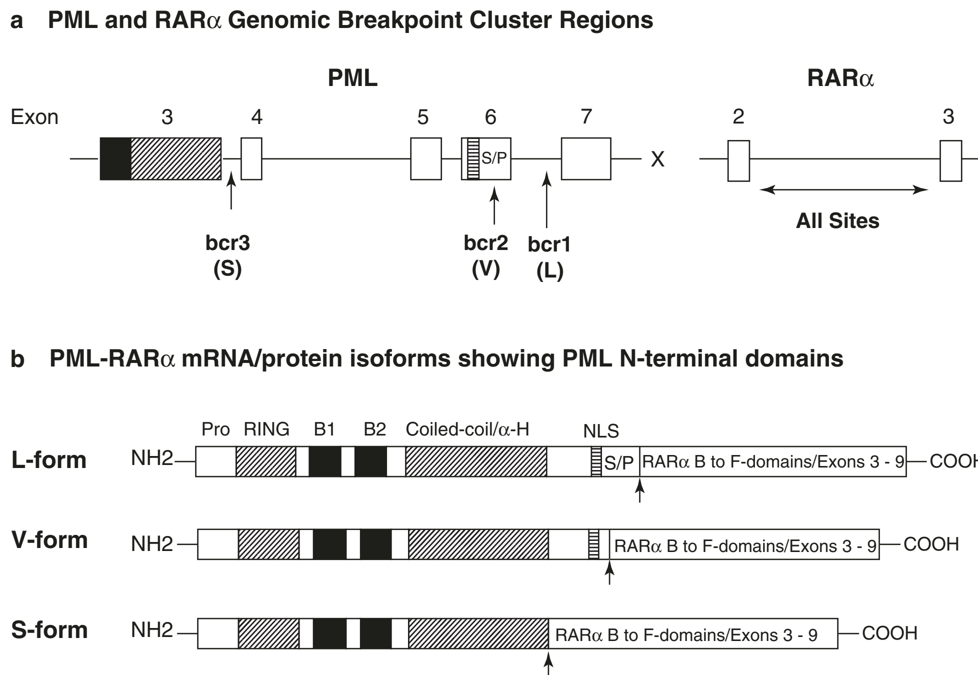


Fig. 21.4 Formation and PML region structure of PML-RARα isoforms. (**a**) DNA-level diagram is limited to the exons of PML and RARα involved in the formation of PML-RARα fusion transcript/protein. *Boxes*, numbered exons; lines, introns. Thin *arrows* indicate the intronic breakpoint cluster region (bcr) sites of DNA breakage that produce the L (bcr1), V (bcr2), and S (bcr3) isoforms of PML-RARα. (**b**) 5′-PML region linked to common RARα segment containing B–F domains shown in Fig. 21.2. The variably filled *rectangles* indicate the mRNA coding/protein regions for the proline-rich (Pro), RING, B-box (B1 and B2), coiled-coil, alpha-helical, and serine-proline-rich (S/P) domains. NLS indicates a nuclear localizing sequence in PML exon 6

amino acid heptad repeats in the coiled-coil region, which serves as a critical interface for the formation of PML homodimers and of heterodimers with PML-RARα in APL cells (see text to come). The RBCC motif, encoded by the first three exons of the PML gene, is present in all of the 18 isoforms generated by differential splicing of the succeeding 6 exons [56].

The physiological function of PML remains rather ill-defined despite many years of intensive investigation [57]. This is partly related to the lack of a compelling phenotype of PML knock-out (PML^{-}/PML^{-}) mice, which appear essentially normal under nonstressed conditions, and partly to the highly pleiotropic and complex functions that have been associated with PML. Massive experimental evidence indicates involvement in fundamental cellular processes, including proliferation, senescence, apoptosis, and differentiation and in pathological processes, including viral infections and oncogenesis. A cardinal early observation was that PML is expressed principally in nuclear structures called nuclear bodies (NB) in a wide variety of cell types [58, 59]. PML NB are quite heterogeneous ranging from 0.2 to 1 μM in size and in number from 1 to 30 per nucleus, where they often are nonrandomly distributed relative to other nuclear elements [57]. From studies using cells derived from PML^{-}/PML^{-} mice, it has been determined that PML expression is a central player in both NB structure and function [60], although there some evidence indicates that alternative NB proteins can also serve in this role [57]. An ever increasing number of proteins have been assigned to PML NBs, a recent review citing greater than 70 proteins and a recent bioinformatics study citing an interactome of 166 proteins [57, 61]. However, only a minority of the PML NB proteins have been demonstrated to directly interact with PML, importantly including a ubiquitin-related enzyme, SUMO-1 (small ubiquitin-related modifier), which produces posttranslational modification of PML required for the recruitment of many other PML NB proteins [62]. Based on the presence of several additional SUMOylation-involved proteins in PML-NBs and the presence of SUMO interaction motifs (SIMs) in the majority of PML interactome proteins, it was postulated that the on–off sumoylation status may provide a binary switch mechanism regulating the location, integrity, and activity of many PML NB components [61]. Recently, it was demonstrated that site-specific sumoylation is a prerequisite for recruitment of the ubiquitin E3 ligase RNF4, leading to degradation of PML by the proteosome [63, 64]. Several other posttranslational modifications, including phosphorylation, acetylation, and ISGylation among others have also been importantly related to PML protein biology [65]. Also, there has been a recent increased effort to dissect the specific activities of the multiple protein isoforms, including several present in the cytoplasm due to translation from mRNA

splice forms lacking the nuclear localizing sequence (NLS) encoded by exon 6.

Pathologically, PML has been defined as a tumor suppressor gene based on many experiments demonstrating an inhibitory effect on cell proliferation and a stimulatory effect on apoptosis [66, 67]. Consistent with this designation, PML knock-out (PML^{-}/PML^{-}) mice have an increased incidence of skin papillomas and lymphomas, many of high grade, after treatment with a chemical mutagen, and cells from PML^{-}/PML^{-} mice are relatively resistant to different types of apoptogenic stimuli. Also consistent decreased levels of PML protein have been demonstrated in human cancer cells [68]. Conversely, an analysis of LIC in a human chronic myeloid leukemia (CML) model in PML^{+}/PML^{+} vs. PML^{-}/PML^{-} mice indicated that greater oncogenicity was associated with higher PML expression [69]. Further, this PML-associated effect was related to the preservation of stem cell/LIC function by inducing replicative quiescence, while PML deficiency was associated with continuous cell cycle entry, eventually leading to replicative exhaustion. Notably, older nonleukemic PML^{-}/PML^{-} mice developed hematopoietic insufficiency, suggesting that one physiological role of PML is to regulate stem cell replication to maintain a lifelong reservoir. These two differing scenarios related to tumor cell behavior are likely representative of the diversity and complexity of PML activity in different cell contexts [65, 67, 70].

Structure and Generation of PML-RARα and RARα-PML

The PML-RARα fusion gene derives in each APL case from breakage of the PML gene on chromosome 15 in one of three breakpoint cluster regions (bcrs) and from breakage of the RARα gene on chromosome 17 in the second intron (Fig. 21.4a, b). PML-RARα bcr1 cases result from genomic DNA breaks in PML intron 6, producing, after mRNA processing, the long(L)-form PML-RARα fusion transcript. In bcr3 cases, the breaksite occurs in PML intron 3, producing the short(S)-form of PML-RARα mRNA. Compared to L-form mRNA, the S-form transcript lacks PML exons 4–6 which primarily encode a proline/serine-rich region with several potential phosphorylation sites. Exon 6 also contains the PML nuclear localizing sequence (NLS) and an important proteolytic site. In bcr2 cases, the PML breaksite occurs at different sites in PML exon 6, which results in deletion of variable amounts of coding sequence from the resultant variable(V)-form PML-RARα fusion transcript. Frequently, additional nucleotides derived from RARα intron 2 are incorporated at the end of the deleted PML 6 exon, which preserves the translational open reading frame (ORF) in all PML-RARα V-form cases [71–73]. Among 221 PML-RARa-positive adult cases, the frequency of L-, S-, and V-form fusion transcripts were 55%, 37%, and 8%, respectively [74]. In pediatric cases, there is a higher frequency of V-form cases, up to 27%, and a proportionate reduction in S-form cases [75, 76].

The reciprocal product of t(15;17), RARα-PML is detected in about 75% of PML-RARα-positive APL cases [77–79]. A recent study demonstrated that a significant portion of the 25% RARα-PML-negative cases can be accounted for by complex rearrangements involving a third gene, by deletions and by alternative mRNA splicing [80].

In order to try to understand the genesis of PML-RARα/RARα-PML fusion gene products, detailed DNA sequence analyses have been performed to identify the precise genomic breaksites. The RARα breaksites can occur throughout the 17 kb-long intron 2, although a few favored microcluster sites were identified, only one of which had an identifiably significant consensus sequence—a high-stringency binding site for the DNA double-strand break repair enzyme topoisomerase II [72, 73]. A similar nondescript pattern prevailed for PML intron breaksites in random APL cases. However, in a subset of APL patients who relapsed after previous treatment with anthracycline topoisomerase II inhibitors for prior cancers or multiple sclerosis, agent-specific (mitoxantrone and epirubicin) strong hotspot breaksites were identified in PML intron 6, and similar but weaker breaksite clusters were found in RARα intron 2 [81–83]. In both PML and RARa, the breaksites contained short homologous sequences suggesting that the fusion gene was generated by means of the nonhomologous end-joining pathway. Of note in these treatment-related APL cases, the median time from drug exposure to leukemia development was about 2 years, which could be consistent with the latency period observed between initial PML-RARα exposure and leukemia development in preclinical models (see text to come). Interestingly, a longer latency period was documented in a spontaneous APL case in a 10-year-old boy by demonstrating the same DNA-level PML and RARα breaksites in the blood Guthrie card obtained at birth [84]. Further study is required to assess whether alternative consensus sequences with no known relationship to DNA break repair proteins found adjacent to RARα intron 2 breaksites in spontaneous APL cases may provide insight into alternative mechanisms of PML-RARα formation [72].

Role of PML-RARα in Leukemogenesis

An early and long-standing concept has been that the primary leukemogenic activity of PML-RARα is due to a dominant-negative inhibitory effect on normal RARα, which blocks RARα-mediated terminal granulocytic differentiation at physiological ATRA concentrations [85, 86]. One basis for this concept was the observation that over-expression of transduced RARα in lineage-negative mouse bone marrow progenitor cells could produce sustained self-renewal and

arrest myeloid differentiation at the promyelocyte stage [87, 88]. These effects could also be produced by RARα with an inactivating mutation in the LBD [88], which had been demonstrated to inhibit ATRA-induced HL-60 cell differentiation by a dominant-negative mechanism [89]. Also supportive was the observation that transduction of PML-RARα into multipotential human hematopoietic progenitor/stem cells (HSC) co-opted the differentiation program, rapidly committing the transduced cells to the neutrophilic granulocyte pathway with arrest at the promyelocyte stage [90]. Two lines of experimentation provided a strong molecular rationale for the concept. First, ATRA exposure was shown to produce selective proteolytic degradation of PML-RARα, which would relieve the dominant-negative inhibition of still intact RARα, unblocking RARα-mediated differentiation [91, 92]. Second, PML-RARα was demonstrated to form a homodimer through the coiled–coiled dimerization interface of the PML region, which could usurp RARE binding sites and recruit a double dose of corepressor–HDAC complex compared to RXR:RAR heterodimers (Fig. 21.5a, b) [93–95]. This could explain the higher, pharmacological concentration of ATRA required to trigger differentiation of APL cells.

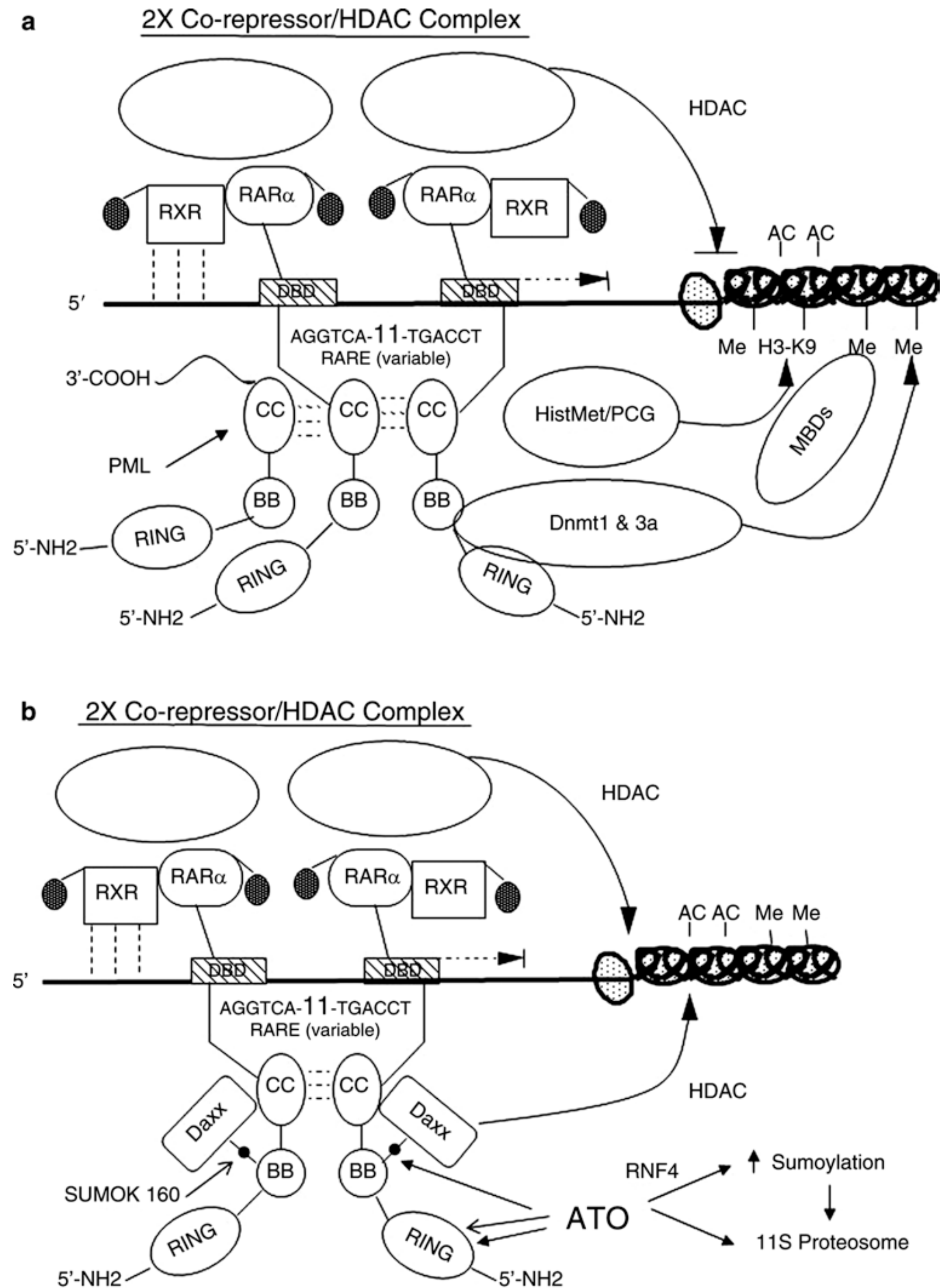


Fig. 21.5 Model illustration of the PML-RARα homodimer-nucleated hetero-oligomeric complex and action sites of arsenic trioxide (ATO). (**a**) PML-RARα homodimer situated on an atypical RARE (consisting of an inverted repeat of the right-side half-site separated from a canonical left-side half-site by 11 base pairs), stabilized by the noncovalent bonding between the two central coiled–coiled (cc) regions (*dashed lines*). Two RXR molecules form part of the complex by interacting with the interaction interface in the E-regions in the RARα portion of PML-RARα. On the lower *left side*, a normal PML molecule forms part of the complex also by interacting with the cc region of PML-RARα. On the *lower right*, several other repressor proteins have been recruited to the complex, including DNA methylases (Dnmt1 and 3a), histone methylases and demethylases (PCG, polycomb group proteins), and DNA methyl-binding proteins (MBDs). (**b**) *Lower left*: The suppressor gene Daxx is bound to the B-Box region (BB) of the PML portion of PML-RARα, which is dependent on sumoylation of lysine 160. *Lower right*: ATO degradation by the proteosome induces increased, polysumoylation of K160 by the ubiquitin E3 ligase RNF4 and also directly binds to and oxidizes vicinal cysteine residues in the PML RBCC region

It could, also, account for the effectiveness of HDAC inhibitors in complementing ATRA activity, which was particularly evident when ATRA-LBD interaction was compromised by an inactivating LBD mutation in PML-RARα [93]. A subsequent series of reports documented the staged recruitment of several additional components to PML-RARa-corepressor complex gene promoters, which are not usually evident or present at lower levels at RXR/RARα promoter sites, that can further modify chromatin structure and the level of transcriptional repression. Among these components are DNA methylases, DNA methyl-binding proteins, and histone methylases and demethylases (associated with polycomb group repressor proteins) [96–100].

This focus on the disruption of RARα-mediated transcriptional activity suggests that the PML region contribution to the fusion gene might be essentially facilitative related to homodimer formation. However, many experiments now indicate that the PML region has an active, even dominant, role in APL leukemogenesis. Most dramatically, in a TM model, a PML-RARα transgene with a mutation in the RARα-region LBD that prevented ATRA binding was equally leukemogenic compared to a nonmutant PML-RARα transgene, while a transgene with the same inactivating mutation in normal RARα was not leukemogenic [101]. These results demonstrate that PML-region activity but not RARα-region transcriptional regulatory activity is required for leukemogenesis. In accord, an artificial recombinant transgene in which an HDAC, the key effector of RARα-mediated corepressor activity (see RARα section), tethered to RARα was not leukemogenic [102]. In meticulous experiments with a variety of naturally occurring and artificially generated forms of X-RARα, it was demonstrated that homodimer formation is obligatory for leukemogenic activity and that PML-RARα homodimers were uniquely potent [103–105]. One way that PML-RARα homodimers have been considered to contribute to leukemogenicity is by interacting with the coiled–coiled region of normal PML, producing dominant-negative inhibition of this important cell regulatory molecule (Fig. 21.5a, b; see PML section) [106, 107]. In agreement, a PML-RARα transgene was more leukemogenic in PML^-/PML^- TM than PML^+/PML^+ TM, while it had an intermediate effect in $PML+/PML^-$ TM [108]. This interaction is also responsible for the signature cytological finding in PML-RARα-positive APL, as revealed by immunofluorescent staining: that PML is dispersed to myriad microspeckles throughout the nucleus in APL cells, rather than localized to discrete PML NBs as in normal cells [109]. Subsequent experiments demonstrated that a requirement for leukemogenesis in addition to the nucleating PML-RARα homodimer is the recruitment of RXR as part of a high molecular weight hetero-oligomeric complex [110–112]. Notably, the RARα region of PML-RARα is required for this interaction, since mutagenization of key amino acid sites in the LBD/E region of PML-RARα eliminates RXR recruitment and leukemogenicity [41]. Overall, these experiments indicate that the hetero-oligomeric complex can act as an inhibitor of both PML, as described previously, and of normal RARα by sequestering its heterodimerization partner RXR, as well as by competing for RARE DNA binding sites. Additional experimental evidence, however, indicates that this double dominant negative mode is still insufficient to explain the role of PML-RARα in the complex pathogenesis of APL.

A shortcoming of this presentation is that it does not consider the dynamics of the leukemogenic process. From several TM model studies a consensus conclusion is that PML-RARα is essential for the initiation of the disease process, but that additional complementary mutations are required for progression to full-blown APL-like leukemia [40]. The fundamental basis for this conclusion is a long latency period from PML-RARα initiation to leukemia development, which occurs in only a fraction of the at-risk mice (incidence, i.e., penetrance, <30%). In many molecular genetic experiments, the latency interval and penetrance fraction have been used as a measure of leukemogenic potency. A variety of evidence supports the presumption that secondary mutations required for disease progression occur during the latency interval, including cytogenetic changes indicative of clonal evolution and the divergence of gene expression patterns in leukemias arising in different TM models [113–115]. Genetic cross-breeding experiments to select TM with haploinsufficiency for genes affecting APL differentiation (*PU.1* and *CEBPA*) [43, 116] and/or other vital APL cell processes (PML; see earlier discussion) demonstrated markedly increased leukemia penetrance, suggesting that endogenous genetic or epigenetic changes that reduce the expression or activity of these molecules could be involved in disease progression. Finally, the co-expression of kinase genes with activating mutations that augment cell proliferation (*FLT3ITD* or oncogenic *RAS* mutations) had a potent effect, both decreasing latency and increasing penetrance [117, 118]. Based on these observations and evidence of cooperativity in human AML between activating kinase gene mutations that primarily affect cell proliferation and various transcription factor fusion gene mutations that primarily affect differentiation, it was proposed that these two types of mutations may be the minimal essential requirements for leukemogenesis [117], Overall, these observations indicate that the leukemogenic activity of PML-RARα must be considered on a temporal basis with respect to the disease process.

The primary leukemia initiating activity (LIA) of PML-RARα has been associated with little initial change in differentiation but, rather, with the acquisition of self-renewal capacity, a key property of LICs. Thus, during the preleukemic phase in TM, no differences in cell phenotype and very

limited gene expression differences were noted between early myeloid cells or promyelocytes from PMR-RARα-expressing TM versus control wild-type mice [115]. Similarly, minimal early phenotypic changes were observed in a more natural "knock-in" mouse model in which a single copy of PML-RARα was inserted into the promoter region of the promyelocyte expression-specific gene cathepsin G (CG) [119]. Two remarkable observations were (1) that there was a marked increase in penetrance (≤20–90%) without a change in latency compared to the corresponding CG-promoted TM model and (2) that this was associated with a very low level of PML-RARα expression (only 3% of that observed in the corresponding TM). Since the expression level of PML-RARα was less than that of normal PML or RARα, it was importantly suggested that a gain of function rather than a dominant-negative mode of action might be involved in the establishment of initiated preleukemic cells (see text to come). Recently, it was demonstrated that the most essential change in PML-RARα-initiated cells from PML-RARα CG-knock-in mice was the acquisition of self-renewal capacity at all levels of the preleukemic myeloid differentiation hierarchy from HSCs up to the promyelocyte stage [42]. There was no expansion of myeloid cell precursors or promyelocytes with self-renewal capacity, only of postreplicative neutrophils, until the leukemic stage was reached, at which time there was a marked increase in granulocyte–monocyte progenitors and promyelocytes, accompanied by normalization of neutrophil levels. Additionally, it was demonstrated that 1 in 100 cells from the leukemic promyelocyte cell population was able to generate transplantable leukemias in syngeneic mice, certifying that these normally short-lived, effete cells had acquired the crucial in vivo self-renewal property of LICs. Similar observations were reported in a TM model [43]. These results lead to at least three important conclusions. First, they indicate that the self-renewal proliferation effect imparted by PML-RARα to initiate the disease process is insufficient to account for the expanded proliferative capacity of the fully developed leukemia cells, which must be complemented by the acquisition of secondary genetic or possibly epigenetic aberrations. Second, the variable levels of differentiation block at the promyelocyte stage in different mouse genetic APL models also indicate a requirement for the acquisition of secondary aberrations to complement the inherent differentiation inhibitory effects of PML-RARα. In this regard, a potential contributory factor might be variations in the level of PML-RARα expression during disease progression [120]. Alternatively, the complementary aberrations, e.g., in oncogenes with kinase activity, might affect PML-RARα transcription complexes in a manner that alters their activity on differentiation-modulating target genes. Third, the documentation in mouse genetic models that a significant subpopulation of leukemic promyelocytes (1:100) has LIC capability suggests that the disease, once established, can be maintained by this expanded cell population, as well as by any antecedent cell in the APL hierarchy targeted by PML-RARα to initiate the disease. If applicable to human disease, this has obvious treatment implications.

As introduced previously, there is now much experimental evidence to indicate that positive gain-of-function activity of PML-RARα is at least as important as its dominant-negative inhibitory activity particularly in disease initiation but also during disease progression. An experimental model that has been very useful in studying how PML-RARα produces gain-of-function activity has been a human myelomonocytic leukemia cell line harboring a transduced and conditionally inducible PML-RARα transgene (called U937PR9) [121]. Switching on this transgene, which mimics the initiation event in APL, results in many changes in the expression of gene transcripts, as measured by oligonucleotide gene expression (RNA) array analyses. Several genes of known importance for myeloid differentiation were downregulated, consistent with the dominant repressor effect of PML-RARα on differentiation. However, in accord with a gain-of-function mode, a number of genes were upregulated, including some critically associated with increased cell proliferation and self-renewal and with reduced DNA repair that might predispose to increased mutagenesis during disease progression [121–123]. Notably, the majority of the regulated genes are not known to have a canonical RARE in the gene promoter, which argues against direct regulation via the RARα region of PML-RARα in the absence or presence of ligand (ATRA). Extensive further investigation has implicated the PML component of the fusion gene in this aberrant gene regulation, essentially, by two different mechanisms. One is based on documentation that the hetero-oligomeric complex nucleated by the PML-RARα homodimer has a markedly relaxed specificity for the sequence, orientation, and spacing of the DNA promoter RARE (Fig. 21.5a, b) [124, 125]. Recent reports document that the expanded repertoire of PML-RARα target genes compared to normal RARα target genes is extensive and, furthermore, that most are associated with epigenetic alterations, including histone acetylation and methylation and DNA methylation, indicative of functional significance [126–129]. A second general mechanism is related to protein:protein interactions of the PML region with other transcription modulators. The precise mechanism has only been documented in only one instance in which two alternative transcription factors were rendered ATRA sensitive by tethering them to the PML region of the fusion gene [130]. However, some variation of this mechanism seems probably related to observations of ATRA-modulated gene expression by PML-RARα but not by normal RARα, which include observations in APL cells of the master myeloid differentiation regulatory genes PU.1 [129, 131], CEBPA [43], and CEBPB [132]. An alternative

variation on this theme is that the dense corepressor complex formed by PML-RARα may indirectly affect the activity of alternative transcription factors by depleting modulating cofactors such as RXRs or HDACs [105]. This indirect mechanism may apply to the modulation of the AP-1 transcription factor composed of Fos and Jun [133, 134], which has been suggested to be of central importance in APL pathogenesis [135, 136].

Finally, based on evidence that sumoylation is critical for PML function (see PML section), a principal lysine sumoylation site (K160) in the B-box domain was mutagenized, and the mutant PML-RARα*K160R* was determined to retain most properties of wild-type PML-RARα but to lack the capacity to block terminal granulocytic differentiation [107]. Notably, PML-RARα*K160R* was able to form oligomeric complexes capable of binding a dense corepressor complex but, like artificial RARα homodimer fusions [103, 104], its "transforming capacity" was limited to hematopoietic cell immortalization in vitro and the generation of a expanded myeloproliferative disorder but not leukemia in an in vivo TM model. It was further demonstrated that full leukemogenic potential could be restored to PML-RARα*K160R* by linkage to the strong corepressor Daxx, previously demonstrated to require sumoylation for association with PML (Fig. 21.5a, b) [107]. Recently, Daxx was demonstrated to directly interact with and inhibit CEBPB [137] which is a key PML-RARα-specific early response gene required for differentiation after ATRA treatment [132]. Thus, the unique interaction of PML-RARα with the additional corepressor Daxx that has specific granulocyte differentiation inhibitory activity not provided by RARα-region-related corepressor complex may provide an explanation for the favored selection of PML-RARα rather than alternative RARα fusion gene partners in naturally occurring human APL [107].

Alternative RARα Genes

Six alternative RARα gene fusion gene partners in addition to PML have been reported in acute myeloid leukemia patients classified as APL by cytological criteria (Table 21.1). In all cases, the generically named X gene in the X-RARα fusion gene transcript is ligated to the beginning of the 5′-end of the third exon of RARα, as in PML-RARα. Thus, the 5′-truncated RARα segment lacks only a portion of the A/B-- region associated with ligand-independent activator function (although retaining the major phosphorylation site, Ser77). This consistent finding strongly implies that RARα is the critical factor in specification of the APL phenotype [34, 138]. As indicated in the previous section, genetic modification experiments surprisingly indicate that impairment of direct transcriptional regulatory activity of the RARα region of PML-RARα does not affect leukemogenic potency [101], but that, alternatively, this is related to mandatory recruitment of the RARα heterodimer partner RXR to the X-RARα high molecular weight hetero-oligomeric complex [110–112]. The mechanism by which recruitment of RXR contributes to the pathogenesis has not been fully determined, but it is a consistent requirement for leukemogenicity of all X-RARαs that have been tested. The recruitment of RXR enhances promoter DNA response element binding, suggesting that the constant finding of RARα in APL fusion genes is related to recruitment of RXR by X-RARα via the RARα region to aberrant RAREs selectively recognized by the X-component. The concept that the X-component adds a critical leukemogenic factor to the X-RARα-nucleated hetero-oligomeric complex is supported by the aforementioned finding that the mutagenization of a single amino acid site in the PML region of PML-RARα that did not disrupt the integrity of the hetero-oligomeric complex lost leukemogenic potential [107]. Nevertheless, the RXR sequestration effect posed by the hetero-oligomeric complex *vis-a-vis* normal RARα and alternative transcription factors, as described earlier, may provide an essential and common contribution to the transformed APL phenotype for all X-RARαs.

PLZF-RARα is the most common and most studied of the alternative X-RARαs (Table 21.1). Two studies indicate that the incidence of PLZF-RARα is <1% of APL cases: (1) in a European study of 611 cytogenetically characterized patients with a centrally evaluated consensus cytological diagnosis of APL, which included 18 PML-RARα-negative patients (2.9%), PLZF-RARα was detected in 11 patients (incidence,

Table 21.1 Alternative retinoic acid receptor-alpha fusion genes in APL

Fusion gene	X-partner name	Chromosome marker	Frequency	Reciprocal	ATRA sensitive	ATO sensitive
PLZF-RARA	Promyelocytic leukemia zinc finger	t(11;17)(q23;q21)	~0.5% of cases	Most	Weak; variable	No
NPM-RARA	Nucleophosmin	t(5;17)(q35;q21)	7 cases	Yes	Yes	Yes
STAT5b-RARA	Signal transducer and activator of transcription 5b	None or der(17)	4 cases	No	No	Not reported
NuMA-RARA	Nuclear mitotic apparatus	t(11;17)(q13;q21)	1 case	No	Yes	Not reported
PRKAR1A-RARA	cAMP-dependent protein kinase regulatory subunit R1alpha	None; FISH:del(17)(q21)	1 case	No	Not reported	Not reported
FIP1L1-RARA	Factor interacting with poly(A) polymerase	t(4;17)(q12;q21)	1 APL 1 JMML	Yes	Yes	Not reported

0.8%) [138], and (2) in 225 patients registered by the Eastern Cooperative Oncology Group (ECOG) to North American Intergroup APL trials with the clinical diagnosis of APL (no central review prior to registration), 22 patients were PML-RARα-negative (9.8%) and 1 patient was PLZF-RARα-positive (0.4%) [139]. In 10/12 of the cited cases, the PLZF-RARα fusion resulted from the reciprocal translocation t(11;17)(q23;q21), and in all cases tested, it was associated with the reciprocal fusion gene RARα-PLZF. In 1 of the 2t(11;17)-negative cases that could be tested, the PLZF-RARα fusion gene resulted from an RARα insertion in chromosome 11 and, accordingly, RARα-PLZF was absent [138, 140]. This distinction is clinically important, because the RARα-PLZF-negative case was responsive to ATRA-therapy, whereas patients with both PLZF-RARα and RARα-PLZF are relatively resistant to ATRA therapy (see clinical sections). More generally, PLZF-RARα APL is the only X-RARα reported to exhibit distinguishing cytological and immunophenotypic features—blasts with regular rather than indented nuclei, increased Pelger-like cells and expression of CD56—from those that can be common to X-RARα and PML-RARα cases [141]. Also, PLZF-RARα-positive APL is associated with a poorer prognosis than PML-RARα-positive APL. This has been ascribed both to insensitivity to ATO [142, 143] and to an inability of ATRA-containing therapy to eliminate APL LICs regardless of differentiation-inducing effect, which seems more profound than that for PML-RARα-positive APL [41, 144].

The molecular basis for the relative insensitivity of PLZF-RARα APL to ATRA therapy was initially linked to the binding of corepressor protein to the POZ domain of the PLZF region in a non-ATRA-releasable manner in addition to the common ATRA-sensitive corepressor binding to the RARα region [93–95, 145]. This conclusion was supported in these studies by in vitro and TM experiments indicating that the ATRA resistance of PLZF-RARα-positive APL cells could be at least partially overcome by co-treatment with inhibitors of HDACs recruited by the corepressors. Subsequent findings, however, have indicated that a more important factor is the absence or presence of the RARα-PLZF reciprocal gene product, as indicated previously. This relates to the structure and function of the PLZF gene, which belongs to a gene family homologous to the Droshophila transcription factor *Krüppel*. These proteins have a motif with nine zinc fingers near the carboxy-terminus that interacts with specific DNA response element sequences of select gene promoters. The PLZF intronic breaksites involved in formation of the reciprocal fusion genes usually provides seven, less frequently six, of the zinc fingers to RARα-PLZF [28, 146]. This protein was demonstrated to counter the repressive activity of normal PLZF on cell cycle progression, at least partly related to activation of the cyclin A2 gene promoter [147]. Similarly, RARα-PLZF countered the PLZF repressor activity on the promoter of CRABP1 producing a marked increase in the expression of this protein, which has been associated with increased retinoid metabolism and resistance [148]. Although it only contains two or three zinc fingers, PLZF-RARα was recently demonstrated to preferentially bind to an expanded repertoire of noncanonical RAREs that includes an APL-associated gene promoter set that is significantly overlapping with that described for PML-RARα binding [122, 125, 126, 144]. It was also demonstrated to specifically interfere with the transcriptional regulatory activity of its normal homolog PLZF [144], analogous to the interference of PML-RARα with normal PML function. A key specific effect was to increase cell proliferation in part by countering the repressive activity of PLZF to activate the cMYC gene promoter [144]. Thus, PLZF-RARα-positive APL resembles PML-RARα-positive APL by the critical contribution of the fusion gene product in countering the regulatory activity of the normal X-component, but it differs in the sense that the reciprocal PLZF-RARα and RARα-PLZF proteins are complementarily involved in the pathogenesis. These features likely account for the resistance of PLZF-RARα/RARα-PLZF APL to ATRA-induced differentiation (variably reported) and, particularly, for the ineffectiveness of ATRA in eliminating the self-renewal capacity of LICs [41, 144].

The other five X-RARαs that have been reported in APL are extremely rare, as listed in order of frequency and/or discovery in Table 21.1. Recurrent cases have been reported for NPM-RARα and STAT5β-RARα but only single cases for the other 3 X-RARαs. The FIP1L1-RARα fusion had previously been reported in a patient with juvenile monomyelocytic leukemia (JMML) [149], and, also, an alternative FIP1L1 fusion gene, FIP1L1-PDGFRA (platelet-derived growth factor receptor-alpha), has been associated with the hypereosinophilic syndrome and chronic eosinophilic leukemia [150]. Notably, the breaksite in FIP1L1 intron 15 in FIP1L1-RARα encoded a protein that could form homodimers, as characteristic of APL-associated fusion genes, while this was not so for FIP1L1-PDGFRA proteins in which the breaksite occurred in earlier introns [33]. Three of the fusion genes in which the X-partner was located outside chromosome 17 resulted from reciprocal translocations (NPM-RARα, NuMA-RARα, and FIP1L1-RARα) [33, 151, 152], while those located with RARα in chromosome 17q resulted from insertions and small deletions with (STAT5β) or without (PRKAR1A) microinversions [31, 32, 153]. Two of three translocation fusion genes were associated with the expression of the reciprocal fusions (RARα-NPM and RARα-FIP1L1); any functional role for each of these remains to be determined. Sensitivity to ATRA-induced differentiation has been established for APL cases derived from all three of the translocation-derived fusion genes [149, 152, 154, 155]. No evidence of ATRA sensitivity was observed in

any of the four STAT5β-RARα APL cases, although only one of these patients was treated with ATRA as a single agent [153]. ATO sensitivity has only been tested in NPM-RARα APL, which, like PML-RARα APL, was reported to be sensitive in both a TM model and a relapse patient [156, 157]. No testing for ATRA or ATO sensitivity related to the PRKAR1A-RARα fusion has been performed, but it was speculated that sensitivity of both of these agents is likely since reduced function of the alpha regulatory subunit (PRKAR1A) would likely result in increased catalytic activity of PKA, which has been linked to increased sensitivity to both agents [32]. More generally, the finding of recurrent cases for NPM-RARα and STAT5β-RARα implies that the X-partners in these fusions, like PML and PLZF, have some important pathogenic role beyond the formation of homodimeric and hetero-oligomeric complexes, as described earlier. Indeed, it seems likely that all of the X-RARαs contribute by as yet undetermined diverse means to the increased self-renewal properties required for LIC activity in the context of the APL phenotype imposed by X-RARα/RXRα structural complexes on a shared subset of gene promoters. Finally, in this conglomerate consideration of alternative fusion genes, it is noted that one enigmatic APL case has been reported in which only RARα-PML and no PML-RARα could be detected at disease presentation [158]. Also, in a rigorously documented set of 611 patients with characteristic features of APL, five patients (0.8%) lacked any rearrangement of RARα [138]. Overall, the previous considerations suggest that additional pathogenic elements of this disease remain to be discovered.

Role of PML-RARα in the Response to Treatment

Both ATRA and ATO, two agents that have selectively potent therapeutic effects in APL compared to other types of leukemia, have the common property of inducing proteolytic degradation of PML-RARα [36, 91]. The simplest and most direct explanation for the therapeutic effect of these agents is that by removing the dominant-negative suppressor effect of PML-RARα, normal RARα and PML (produced by the non-translocated gene loci) can re-establish physiological pathways leading to granulocyte terminal differentiation and/or apoptosis [91, 159, 160]. Although this may provide a partial explanation, the experimental details indicate that the molecular response is more complex, as well as different, for the two agents.

Exposure of APL cells to ATRA is succeeded by changes in the regulation of many hundreds of genes, leading to discernible differentiation within 24–48 h associated with a temporary increase in resistance to apoptosis [121, 126, 161–165]. The "first wave" of these reported changes in gene transcript levels occurs within 6 h of ATRA exposure [126, 165], before there has been substantial degradation of PML-RARα [91, 92]. Among these early ATRA response genes, there is no enrichment of gene promoters containing canonical RAREs required for RARα-mediated transcription, while there is some enrichment of those with atypical RAREs that can be recognized by PML-RARα [126]. Further, there were many more genes regulated in response to ATRA in U937PR9 cells (defined earlier) previously induced to express PML-RARα than in control U937 cells containing endogenous RARα [126]. These observations provide incontrovertible evidence that at least the early transcription response to ATRA is mediated predominantly by PML-RARα, not RARα. The crucial importance of PML-RARα as the primary mediator of ATRA activity in APL is also supported by the observation that after clinical relapse from ATRA-containing therapy, inactivating mutations that develop in the RARα LBD in association with acquired ATRA resistance are invariably in PML-RARα, not RARα [166–168]. Many of the early ATRA response genes are regulated in the opposite direction from that observed after PML-RARα induction in U937PR9 cells with approximately equal numbers of genes being changed from downregulated by PML-RARα to upregulated by ATRA and vice versa [122, 126]. Several important functional classes of genes are selectively regulated, including the upregulation of several key transcription factors involved in terminal granulocyte differentiation (CEBPs, PU.1, ID1&2). PU.1 is a recognized master transcription regulator of differentiation processes in hematopoietic cells, and, when it is experimentally manipulated to be switched off or on in APL cells, it has the corresponding effects of inhibiting or promoting terminal granulocyte differentiation [131]. The transcription of the PU.1 gene is activated by the binding to its promoter region of two other transcription factors, CEBPB and OCT-1 [131]. CEBPB transcription is upregulated in APL cells within 1 h of ATRA exposure, and, as previously mentioned, is activated by PML-RARα but not RARα and is inhibited by the PML-associated gene Daxx [132, 137]. This suggests a cascade activation process involving CEBPB relief from Daxx-mediated repression at an early stage, which might then be propagated by activated PU.1, a possibility supported by the finding of coincident PU.1 binding sites and nearby PML-RARα potential binding half-sites in hundreds of gene loci [129]. A key PU.1 target gene in such a cascade may be CEBPE—which as a single upregulated gene can drive terminal granulocyte differentiation in TM APL cells—since a recent report suggests that PU.1 is its principal transcriptional regulator in response to ATRA despite the presence of a classical RARE in the CEPBE gene promoter [169, 170]. Although the exact mechanism of transcriptional regulation is not known, evidence is presented for the recruitment of the histone acetyltransferase p300 to the CEBPE promoter

[170]. This observation is consistent with another recent genome-wide assessment of early epigenetic changes after exposure of APL cells to ATRA (24 h), which demonstrated marked changes in histone acetylation but not in histone or DNA methylation [128]. Thus, these findings lead to the conclusion that the early response of APL cell response to ATRA does not occur by the long-held concept that it relieves dominant negative inhibition of direct RARα-regulated genes essential for terminal granulocyte differentiation. Rather, they indicate that the RARα region of the fusion gene provides ATRA sensitivity to PML-RARα via the PML region, which affects a wide array of target genes either directly or indirectly in a gain-of-function manner.

After 6 h of ATRA exposure there is progressive degradation of PML-RARα, and, although there is considerable variation in quantitative estimates between laboratories using the NB4 cell line, this is substantial by 12–24 h and virtually complete by 48–96 h [91, 92, 159, 171, 172]. This process has been attributed to both ubiquitin/proteosome and caspase proteolytic activities directed at the RARα region of the fusion protein, although neither is sufficient for complete degradation [92, 172]. Additionally, evidence has been presented for proteolysis directed at the PML region through an alternative process called ISGylation initiated by early ATRA activation of the ubiquitin-like E1 ligase UBE1L [173]. More global proteomic studies during this ATRA exposure interval have demonstrated the associated regulation of protein systems, particularly involving signal transduction (altered), ubiquitin/proteosome activity (increased), cell cycle progression (decreased), RNA metabolism (altered), and protein synthesis (decreased) [165, 174–176]. However, reported events during this post-ATRA interval are complex and sometimes apparently contradictory, e.g., a proteomics study reported the physical downregulation of protein translation initiation factors [174], while another study focusing on phosphorylation signaling reported an increase in translational initiation [177]. Notably, at the mRNA level PML-RARα and RARα transcripts are maintained, despite degradation at the protein level [92]. Concurrently, many continuing and new gene transcript level changes are observed between 6 and 48 h, several prominently affecting cell signaling involving calcium and interferon [165]. The complex gene networks involved in executing the steps to final terminal differentiation, including a “third wave” of gene transcription regulation involving terminal differentiation marker and functional genes [165], is poorly understood. This may partially involve regulation by RARα after relief of dominant negative suppression by degradation of PML-RARα, but it likely involves other regulatory mechanisms activated during the process as well [163, 178].

The molecular mechanisms that determine the clinical response of APL cells to ATO have been less precisely defined than for ATRA. This is partly related to the complex, pleiotropic activities of ATO that can vary depending on ambient conditions [179, 180] and partly to uncertainties about translating clinically effective ATO concentrations to in vitro studies. An initial study identified dual dose-dependent effects using both fresh APL cells in short-term culture and the NB4 cell line: at ≤0.5 μM ATO, the predominant effect was the induction of partial, atypical differentiation; at >0.5 μM, the predominant effect was the induction of apoptosis [14, 181]. The probable relevance of both types of activity was supported by pharmacokinetic (PK) analyses in ATO-treated APL patients demonstrating peak plasma concentrations of total arsenic >5 μM with a biphasic excretion profile over 24 h to near basal levels [182]. However, more recent studies using more advanced technologies capable of identifying the active trivalent arsenite form, as well as the oxidized, pentavalent arsenate form and methylated metabolites, indicate that very transient peak levels of total arsenic are almost always <1 μM and the arsenite form typically <0.5 μM after standard therapy with 0.15 mg/kg ATO intravenously over a 2-h period [183, 184]. During daily treatment, there is some enrichment of the methylated metabolites, which are not effective differentiation inducers but have a greater apoptotic effect than arsenite at 0.5 μM [185]. Of note, the highest concentration of arsenic is associated with the cellular fraction, primarily with erythrocytes bound to hemoglobin [186]. Also, there is substantial ATO concentration in some cell types, especially keratinized cells such as nails and hair [182], but the level in APL cells under clinical treatment conditions has not been defined. The clinical response of patients to standard ATO therapy strongly suggests that lower ATO concentrations (<0.5 μM) are most relevant and further suggests that in vitro studies conducted at much higher ATO concentrations, e.g., >1 μM, may have limited clinical relevance. As originally described, the typical clinical response consists of the appearance of partially differentiated myeloid cells admixed with apoptotic cells in the peripheral blood, frequently appearing at increased levels more than a week after initiating treatment [14, 187]. Whether the circulating apoptotic cells are derived from the differentiated cells, as occurs following ATRA-induced differentiation of APL cells [163], or by an independent process has not been determined.

As previously noted, a primary finding was the degradation of PML-RARα (and PML but not RARα) within a few hours after ATO exposure associated with the reformation of aggregated PML nuclear bodies [36, 160]. Degradation was noted at 0.1 μM ATO, but this occurs much more slowly at this low concentration than at 1 μM [160, 181]. A series of important discoveries have been made about the details of this process. First, it was demonstrated that ATO treatment results in the sumoylation of lysine residues in the PML region of PML-RARa [188], Second, it was demonstrated that sumoylation specifically of lysine-160 (K160) is required

for recruitment along with sumoylated PML to reconstituted mature PML nuclear bodies where proteosome-dependent degradation occurs [189]. Third, it was found that polysumoylation of K160 via its SUMO interaction motifs (SIMs) recruits the RING finger ubiquitin E3 ligase RNF4, thus, defining a novel polySUMO-dependent ubiquitin-mediated proteolysis mechanism [63, 64]. Finally, it was demonstrated that within 10 min of exposure, ATO is bound to specific cysteine residues in two zinc finger motifs of the RBCC domain of the PML region [190]. Evidence was provided for the formation of octomer PML-RARα/PML complexes, including homodimers due to cross-linking between the RBCC regions of two PML-RARα/PML molecules, which could explain the rapid, aggregated PML nuclear body formation and enhanced polysumoylation required for proteosomal degradation [190].

Despite the informative detail, important questions remain about the relationship of PML-RARα degradation to the cell biological activity of ATO in APL. Is PML-RARα degradation *required* for the differentiation or apoptotic response of APL blasts to ATO? One set of experiments suggests that this could be so for differentiation: primary hematopoietic cells transduced either with the nondegradable K160-mutant PML-RARα or mutated dominant-negative RNF4 could not be induced to differentiate by 1 μM ATO, while this did occur with wild-type controls [63]. In this system, differentiation would presumably be mediated by physiological ATRA concentrations via normal RARα, which is not degraded by ATO [160], after the removal of PML-RARα dominant-negative inhibition. However, after treatment of NB4 cells with 0.1 μM ATO, the early postexposure (6 h) gene expression profile closely resembled, albeit at a lower level, that induced by ATRA [165, 181], which is primarily mediated by PML-RARα, as discussed previously. Notably, this low ATO concentration was surprisingly effective in dissociating corepressor protein (SMRT) from PML-RARα, which was attributed to SMRT phosphorylation secondary to ATO-mediated activation of the mitogen-activated protein (MAP) kinase MEK-1 [191]. Although the role of MEK-1 is controversial [192], dissociation of the corepressor in combination with delayed PML-RARα degradation at low ATO concentrations [181, 191] could result in the activation of aberrant PML-RARα-regulated gene promoters at endogenous ATRA levels [124, 125]. These results in different cell systems indicate that further studies are needed to understand how ATO mediates APL blast cell differentiation, particularly as observed in vivo. Assessment of the requirement of PML-RARα degradation for APL cell apoptosis is even more difficult, because at ≥1 μM concentrations ATO can induce apoptosis in many cell types that lack PML-RARα [193]. Thus, an alternative question is: does the presence of PML-RARα sensitize APL cells to ATO-induced apoptosis? Attempts to address this question have produced controversial results [188, 194], but a recent report suggests an intriguing mechanism by which this might occur (see text to come) [195]. Finally, is ATO-induced PML-RARα degradation *sufficient* to produce differentiation and/or apoptosis? The answer to this question is a definitive no, since studies of ATO-resistant NB4 cells effectively degrade PML-RARα without undergoing apoptosis or differentiation in response to ATO [194, 196].

Numerous studies have been performed to attempt to unravel how ATO produces apoptosis in APL cells regardless of PML-RARα contribution [179, 180]. Arsenite produces oxidative stress by binding to and decreasing the reducing capacity of the tripeptide GSH, the major intracellular buffer for reactive oxygen species (ROS), and by binding to vicinal, i.e., neighboring, sulfhydryl (thiol) groups in cysteine residues of redox-sensitive proteins [180, 197]. The relatively high sensitivity of APL cells to ATO-induced apoptosis has been attributed to low endogenous levels both of GSH and of enzymes (glutathione peroxidase, catalase, and glutathione-S-transferase) involved in regulating superoxide/free radical production from H_2O_2 [171, 198]. The importance of the GSH system in ATO sensitivity was experimentally demonstrated: increasing reduced GSH/sulfhydryl levels decreased ATO sensitivity and vice versa. Also, increased GSH levels were present in NB4 sublines selected for ATO resistance to which ATO sensitivity could be restored by depleting sulfhydryl levels [194, 198]. It was additionally proposed that postexposure ATO binding to vicinal thiols might inhibit H_2O_2-regulatory enzymes and augment endogenous APL sensitivity [171]. However, these enzymes apparently do not directly bind ATO at clinically relevant concentrations (≤1 μM), while this has been demonstrated for thioredoxin reductase (TrxR), a key regulatory enzyme in the alternative thioredoxin (Trx) ROS buffer system [199]. The oxidized (disulfide) forms of both GSH and Trx require NADPH as a reducing substrate (Fig. 21.6). Thus, it is of interest that after several days' exposure of NB4 cells to ATO at a concentration reported not to produce differentiation in these experiments (0.75 μM), gene expression analysis showed a remarkable selective increase in transcripts involved in neutrophil oxidant production, including several components of NADPH oxidase [200]. Further, this was identified as the main source of ROS. Subsequent studies found that NADPH-derived ROS are also increased in untreated NB4 cells and that aberrant regulation of NADPH oxidase is related to impairment of cyclic adenosine monophosphate (cAMP) signaling by PML-RARα [195, 201]. Although no mechanistic details about the impairment link were provided, this scenario seems a reasonable extension of the established inverse relationship of cAMP levels to NADPH oxidase activity in mature neutrophils, which is the principal source of superoxide generation in the antimicrobial response [202]. These studies implicating increased

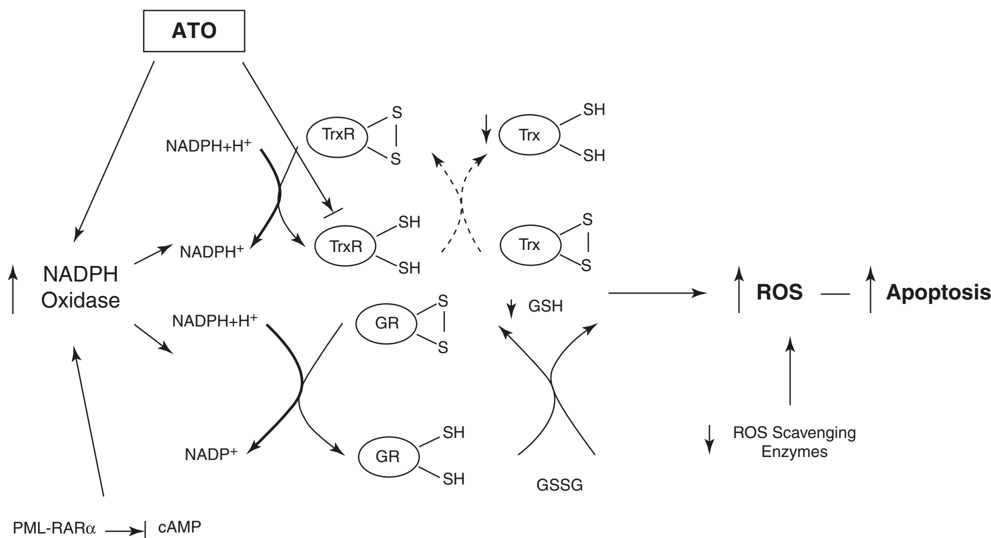


Fig. 21.6 Suggested pathways for the enhanced sensitivity of APL cells to ATO-induced apoptosis. APL promyelocytes, as committed neutrophilic lineage cells, have some increased basal levels of NADPH oxidase, a multicomponent enzyme complex involved in the antimicrobial activity of mature neutrophils. This may be enhanced by the inhibitory activity of PML-RARα on cAMP, which stimulates physiologic NADPH oxidase activity. Some studies also indicate that APL cells have constitutively diminished levels of glutathione (GSH) and reactive oxygen species (ROS) scavenging enzymes. Recent studies indicate that shortly after ATO exposure of APL cells, there are further increases in NADPH oxidase enzyme components associated with a demonstrable increase in the oxidative state. This has been related to depleted levels of NADPH, which is the principal substrate for maintaining the two main cellular antioxidant buffer molecules, GSH and thioredoxin (Trx), in an active, reduced state. ATO has also been demonstrated to strongly inhibit the key Trx system enzyme thioredoxin reductase (TrxR), further contributing to increased oxidative state that triggers the cascade of molecular events leading to apoptosis

ROS *production* due to NADPH depletion, principally after ATO treatment and specifically related to the neutrophilic lineage of APL cells, could supersede the importance of earlier-reported sources of ATO sensitization due to increased oxidative stress attributed to deficiencies in ROS scavenging with consequent decreased ROS *removal*. Regardless of attribution, the increased oxidative state generated by ATO has been linked to activation of a number of signaling pathways leading directly or indirectly to apoptosis mediated by a decrease in mitochondrial membrane potential and executed by activation of the classical caspase 3 pathway [171, 180]. Of particular note is a reportedly essential link of activation of the stress (SAPK/JNK) kinase pathway to apoptosis, possibly related to redox-sensitive conformational changes in glutathione-S-transferase pi [203, 204]. Many other potential effects due to direct interactions of ATO with vicinal sulfhydryl groups in redox sensitive proteins, e.g., with cysteines in zinc finger transcription factors, e.g., Sp1 [205], could be contributory to ATO activity, although this would presumably not be APL specific [180]. In summary, the mechanisms accounting for the high sensitivity of APL cells to ATO-induced apoptosis are complex, may involve complementary aberrations in ROS removing and producing systems, may have some PML-RARα-dependent component, can be modulated by ambient conditions that affect the APL cellular redox state—and, as mentioned before, are of uncertain importance to the clinical response of APL to ATO therapy.

Since the specific, high-sensitivity biological activities of ATRA and ATO in APL cells are dependent on differing interactions with the common target gene PML-RARα, these agents might reasonably be expected to have at least additive complementary activity. However, great variability in combined effects on differentiation and apoptosis in vitro have been experimentally demonstrated, including strong inhibitory interactions, using both sensitive and ATRA- or ATO-resistant APL cell lines and fresh APL cells [196, 206–208]. The variability is at least partly related to differing drug concentrations and schedule effects, as well as to variations in ambient conditions, each of which might affect the time-sensitive availability of PML-RARα and other protein mediators related to the differing activity kinetics of the two agents. In the only global gene transcript/protein expression study in which relatively low, differentiation-inducing concentrations of ATRA (0.1 μM) and ATO (0.5 μM) were concurrently applied to NB4 cells, the changes most closely resembled those of ATRA alone with synergistic upregulation of transcripts involved in protein degradation by the ubiquitin/proteosome system and downregulation of proteins involved in translation [165]. In contrast to the variability in vitro, in vivo studies of combined ATRA/ATO treatment in various mouse APL models have almost uniformly demonstrated positive, often strongly synergistic, antileukemia activity [143, 207, 209, 210]. A possible explanation for this difference with profound implications for the treatment of APL was recently published [41]. In this study, it was first

shown in PML-RARα-transduced mixed hematopoietic progenitor cell assays that, although a relatively low concentration of ATRA (0.1 μM) induced terminal granulocytic differentiation, it did not eliminate a subpopulation of progenitor cells that retained clonogenic replating capability, i.e., replicative self-renewal capacity. This in vitro observation suggested that there might be a dissociation of the ability of ATRA to induce differentiation in the bulk of APL cells from an inability to extinguish APL LICs that maintain propagation of the disease. In a subsequent series of in vivo experiments using various APL mouse transplantation models and selected PML-RARα mutations and leukemia initiated by the alternative APL fusion gene PLZF-RARα, further strong evidence was developed in support of dissociation of the differentiation-inducing capacity of ATRA from its anti-LIC activity. Only the latter was able to produce disease cure in the mouse models, using ATRA alone at higher concentrations (≥1 μM) or, most effectively, in combination with ATO and/or cAMP analogs. Molecularly, curative anti-LIC treatment was linked to degradation of PML-RARα under various experimental conditions, including strong inhibition in vivo of synergistic combined ATRA/ATO activity by the proteosome inhibitor bortezomib [41]. The nature of the anti-LIC activity and exactly how it is linked to PML-RARα degradation could not be determined by the functional assays used in these experiments. This must await the identification and characterization of the APL LIC, which, as previously mentioned, appear to present at about a 1:100 level in isolated APL blasts with which they may share many phenotypic properties [42, 43]. Also, these experiments do not exclude a role for ATRA-mediated transcription or distinct ATRA or ATO anti-LIC activities, which could be heterogeneous in different APL cases, partly dependent on the level of hematopoietic progenitor initiated by PML-RARα [211, 212]. The results of these experiments are, however, quite consistent with clinical observations of the curative activity of sustained high-dose ATRA in liposomal form and of combined ATRA and ATO in human APL trials (see clinical sections).

Molecular Mechanisms of Treatment Resistance to ATRA and ATO

Excluding early death from advanced disease or treatment complications, failure to achieve initial clinical remission in patients with de novo PML-RARα-positive APL has rarely been reported after treatment with ATRA [168]. If this is observed, a possible explanation is leukemic chimerism, i.e., the co-existence of APL and a second type of non-ATRA-sensitive leukemia, most frequently t(8;21) AML [213]. The high sensitivity of molecular diagnostic assays for PML-RARα has the potential of masking such chimeras if confirmatory methods such as standard cytogenetics or FISH are not employed. Another documented cause of primary ATRA-resistant disease, reported in 2V-form PML-RARα cases, is a long in-frame insertion of a sequence from RARα intron 2 at the 3′-end of a markedly truncated PML exon 6 that encodes a binding site for additional corepressor protein [71, 72]. One of these cases, additionally, had a frame-shift mutation in the normal PML gene that encoded a truncated carboxy-terminus, deleting the nuclear localizing sequence, which may have also contributed to the ATRA resistance [214]. Combined treatment of ATRA with chemotherapy and/or ATO, as is now common practice, might either overcome or forestall the manifestation of less ATRA-sensitive disease.

The development of acquired ATRA resistance was virtually universal in early clinical studies using ATRA as a single, continuous agent or with low dose chemotherapy [19, 215, 216]. However, some repeat remissions could be secured with ATRA alone if relapse/re-treatment occurred several months after discontinuing ATRA, suggesting a partially reversible systemic mechanism of ATRA resistance [217]. This was substantiated by the pharmacokinetic finding of an ATRA-induced hypercatabolic state that could diminish achievable ATRA plasma levels by up to 80% [218]. The hypercatabolic response was attributed, at least in part, to an increase in liver enzymes that could principally account for the associated increase in oxidative metabolites of orally administered ATRA and, also, to an increase in cellular retinoic acid binding protein (CRABP) in skin that could sequester ATRA to reduce systemic levels. Subsequently, it was found that the hypercatabolic resistance mechanism can be largely avoided by administering ATRA intermittently rather than continuously [219], which has become the standard schedule for administering ATRA after remission induction. A logical deduction of this systemic ATRA resistance mechanism is that alternative agents not subject to its elements should be able to induce second remissions. However, three other retinoid formulations, each with some of these characteristics—oral 9-cis retinoic acid, oral Am80, and intravenous liposomal ATRA [220–222]—were unable to induce second remissions in the majority of ATRA-resistant patients. Overall, these results suggest that other resistance mechanisms are also operative.

By analogy to drug resistance mechanisms in other malignancies, the most probable alternative source of acquired ATRA resistance is an endogenous APL cell mechanism(s). Consistent with this hypothesis, variable loss of sensitivity to ATRA-induced differentiation of fresh APL blasts from patients who relapsed after ATRA treatment in short-term tissue culture has been demonstrated in a majority of cases [167]. Several potential mechanisms of APL cellular ATRA resistance have been proposed, primarily based on investigations of established APL cell lines selected for resistance to ATRA, the most frequent of which is the clonal emergence of cells with mutations in the LBD of the RARα region of PML-RARα [168, 223]. This is, indeed, the only mechanism

of ATRA resistance so far demonstrated in APL patients who relapse after clinical ATRA therapy [166, 167]. Such mutations have been documented in 30–40% of patients at first relapse after ATRA-containing therapy, including cases of relapse long after the discontinuation of ATRA and after intervening chemotherapy [167, 224, 225]. A higher incidence of LBD mutations was observed after relapse from two or more treatment regimens containing ATRA [226]. Consistent with a functional role in ATRA resistance, the LBD mutations increase the transcriptional repressor activity of unliganded PML-RARα and diminish ATRA binding and/or its transcriptional activation [226, 227]. Although some of the mutations might be predicted to have an effect on the ability of ATRA to target PML-RARα for degradation, no specific information is available about this potential effect. Also, the downstream defects effected by the mutations in PML-RARα and their role in disease progression and relapse remain to be elucidated. Whatever, the exact mechanism, the high salvage therapy rate with ATO treatment suggests that this agent, in contrast to chemotherapy, can overcome the APL cellular resistance mechanism(s) associated with LBD mutations in many cases, although specific LBD mutations may be less ATO sensitive [225].

A second major hypothesis has been that ATRA resistance is related to altered metabolism of ATRA within APL cells (reviewed in [168]). An initial study suggested that an ATRA-triggered increase in CRABP2, which has an RARE in its gene promoter, could enhance intracellular sequestration and degradation of ATRA, reducing its effectiveness in inducing APL cell differentiation [228]. However, this result was not confirmed in a larger study, including relapse patients [229]. Further, in contrast to the negative effect of CRABP1 on ATRA activity by increasing ATRA catabolism [230], CRABP2 was subsequently determined to have a positive effect on ATRA activity by facilitating its delivery to nuclear receptors [231, 232]. To date, no evidence has been forthcoming in PML-RARα-positive APL to suggest a role of CRABP1 in ATRA resistance, as described earlier for PLZF-RARα/RARα-PLZF-positive APL [148]. Recently, however, a link was made between the ATRA-specific P450 catabolic enzyme, CYP26A1, and ATRA resistance [233]. Surprisingly, it was found that low sensitivity to ATRA-induced differentiation and to retinoid inhibition of NB4 clonal cell growth was associated with *decreased* levels of CYP26A1. It was postulated that the increased nuclear levels of ATRA found in low CYP26A1-expressing cells would increase selection pressure for ATRA-resistant cells, although the downstream resistance mechanism remained to be defined [233]. Notably, this conclusion seems at odds with the previously cited study indicating that high intracellular levels of ATRA are necessary to inhibit the self-renewal capacity of APL LIC and to effect disease cure [41]. In summary, although an attractive idea, a mechanistic role of variations in intracellular APL cell ATRA concentrations and metabolism in clinical ATRA resistance remains uncertain.

Two other potentially important mechanisms of ATRA resistance have been identified in two well-characterized sublines of the NB4 cell line after selection for ATRA resistance in vitro. In subline NB4.007/6, ATRA resistance was related to constitutive activation of the proteosome and resultant degradation of PML-RARα protein [234]. Sensitivity to ATRA could be partially restored by inhibition of proteosome activity and fully restored by forced expression of PML-RARα but not RARα, supporting other evidence that PML-RARα is required for the ATRA-mediated differentiation response. In NB4-MR2, a subcloned derivative of ATRA-resistant NB4 cells, an increased level of topoisomerase 2-beta (TOP2B) was demonstrated to decrease ATRA-mediated gene transcription and granulocytic differentiation by associating with and increasing suppression by the unliganded PML-RARα/corepressor complex [235]. The TOP2B suppressive activity was subsequently attributed to increased protein stability under regulation of protein kinase C-delta (PRKCD), the most abundant isoform of PRKC in hematopoietic cells [236]. Sensitivity to ATRA-induced differentiation could be restored in the NB4-MR2 cells by inhibiting PRKCD, an enzyme itself regulated by phosphorylation, suggesting the possible involvement of yet another upstream kinase in the pathway, possibly p38 MAP kinase. It is noted, however, that some apparently conflicting evidence regarding the role of PRKCD (and of p38 MAP kinase) in wild-type NB4 cell differentiation has been presented [237, 238]. Although neither of these in vitro ATRA-resistance mechanisms has been demonstrated in clinical circumstances, similarities to the second mechanism have been reported in other cancers. For example, ATRA resistance due to diminished ATRA-mediated RARα transcription has been linked to association with the corepressor complex by the suppressor proteins xeroderma pigmentosa-associated protein Xab2 and the tumor-associated protein Ski [239, 240]. Also, increased activation of protein kinases that can affect the activity of RARα or its transcriptional cofactors by phosphorylation, e.g., PI3K/Akt pathway enzyme components, has been implicated in ATRA resistance [241, 242].

In contrast to ATRA resistance, meager information exists about resistance to ATO under clinical conditions. From the single-agent ATO trials conducted in India, Iran, and China (see clinical sections), there is insufficient documentation to assess whether any of the patients who fail to achieve initial remission have primary refractory disease. The incidence of acquired resistance is, however, clearly much less frequent than for ATRA, since most patients with de novo disease sustain prolonged remissions with probable disease cure, even though optimal single-agent therapy has yet to be defined

[243–245]. Of these patients who relapse, almost all patients can achieve a second remission with ATO alone or in combination with other agents [246–248]. However, the second relapse rate is high associated with a significant incidence of ATO-refractory disease [243], implying the acquisition of ATO resistance. In the best documented trial with long-term follow-up, the great majority of patients who relapsed originally presented with a high APL blast count [244], which implies that acquired ATO resistance has an APL cellular basis rather than a systemic pharmacological basis. On the other hand, the observation in this study that patients with liver toxicity had superior disease-free survival raises the possibility that a pharmacogenetic factor could produce variability in ATO exposure and in the extent of response to treatment. Although no specific molecular mechanism of clinical ATO resistance has yet been identified, the recent finding of a high level of telomerase activity and telomere shortening in first relapse post-ATO treatment APL cells implies the presence of other acquired molecular abnormalities associated with disease progression [247].

In vitro studies, principally of ATO-resistant sublines of NB4 cells, have identified several molecular pathway abnormalities associated with resistance to ATO-induced apoptosis. Increased baseline levels of GSH, which can buffer the increased oxidative stress required for ATO-induced apoptosis, have been found in some ATO-resistant NB4 sublines [194, 249]. After the administration of ATO, however, the differential impact of GSH abnormalities in ATO-resistant cells was related to a more complex effect of the ratio of the reduced and oxidized forms (GSH::GSSG) on redox-sensitive proteins than control activation of JUN kinase-mediated apoptosis [204]. In another NB4 subline, ATO resistance was primarily related to increased activation of the MAP kinase ERK1/2-pathway with consequent inhibitory phosphorylation of the pro-apoptotic protein Bad [192]. In other studies, ATO resistance was related to activation of the PI3K/Akt pathway with secondary effects on the cellular redox state and modification of BCL-family proteins [250, 251]. ATO-sensitivity/resistance has also been related to the expression of the putative arsenic influx transporter aquaglyceroporin (AQP9; high expression with sensitivity) [252, 253], to the arsenic export transporter MRP1/ACBB1 (increase with resistance) [249], and of the metalloid-binding proteins, metallothioneins (increase with resistance) [254]. Detailed analysis in yeast has demonstrated ATO resistance to be a multifactorial complex process [253, 255, 256]. Overall, the in vitro results suggest that, if clinical ATO resistance in APL indeed involves reduced apoptosis, the mechanism is likely to be heterogeneous in different relapse cases and, not unlikely, multifactorial in individual relapse cases reflecting the pleiotropic nature of the agent.

APL Molecular Biology: Clinical Applications

The most essential clinical application of molecular biological knowledge in APL has been to provide a definitive diagnosis. Demonstration of the PML-RARα fusion gene (or one of the rare alternative X-RARα fusion genes) to make a genetic diagnosis is now the accepted standard requirement for certifying the diagnosis of APL [257]. The t(15;17) is also entirely specific for APL but this reciprocal translocation is not manifest by standard cytogenetic methods in up to 20% of PML-RARα-positive cases in which the gene rearrangement occurs either because insufficient APL cell metaphases are present or because more subtle intrachromosomal mechanisms are involved [138]. Fluorescence in situ hybridization (FISH) analysis using RARα- and PML-specific probes, which is not dependent on cell division, has a much lower incidence of false negative results and can provide useful supplementary information, particularly in cases with an atypical or complex derivation of PML-RARα. Another specific method, which is useful for rapidly confirming a suspected diagnosis of APL based on cytological evaluation, is a fluorescein-labeled anti-PML antibody to demonstrate the pathognomonic microspeckles in APL cells due to the displacement of PML from nuclear bodies by PML-RARα [258]. Although not entirely specific, immunophenotyping can also be valuable not only by providing early criteria for or against a suspected diagnosis but also for evaluating phenotypic variation among APL patients (see Chap. 17) [259].

A second clinical application of molecular biology relates to studies assessing the potential prognostic value of molecular markers. Most abundantly this has involved analyses of the three types of PML-RARα, the L, V, and S isoforms, which are revealed in the process of making the molecular genetic diagnosis of APL by reverse transcriptase-polymerase chain reaction (RT-PCR) procedure (Fig. 21.4a, b). Some studies have provided evidence that the minor V-form set of APL patients (3–10%) can be associated with potentially adverse risk or response factors, including higher WBC count and decreased ATRA sensitivity [72, 74, 260, 261], or with a high early relapse rate [71, 262]. However, the small number of these cases has prevented an adequate statistical assessment of potentially increased risk, which, in any event, has likely been eliminated by the increased efficacy of current treatment regimens. Much greater evidence has been presented for an association of the S-form patient set compared to the L (or combined L/V-form) patient set with adverse risk factors, including high WBC count, the microgranular (M3v) phenotype, and with internal tandem duplication mutations of the Fms-like tyrosine kinase-3 (FLT3/ITD; see text to come) [74, 261–264]. However, in most major clinical trial reports, the S isoform has not demonstrated statistical significance as a prognostic indicator of

clinical outcome independently of its association with a high WBC count [74, 265–270]. With the advent of quantitative RT-PCR procedures (RQ-PCR; described next), prognostic significance has been associated with the level of the PML-RARα transcript but this has been quite variable: in two reports, an adverse prognosis was associated with high levels [268, 271], in a third study this was associated with low levels [269] and in a fourth study, the level had no prognostic significance [272]. Technical differences likely account for these variations, including differences in housekeeping genes controls and in the adjustments in raw data made to calculate the normalized PML-RARα copy number.

The only other specific gene structural variation recurrently tested for prognostic significance in APL clinical trials is mutation of the membrane receptor tyrosine kinase *FLT3* by the in-reading-frame insertion of variable numbers of internal tandem duplications of a juxtamembrane coding segment (FLT/ITD mutations). These mutations result in Flt3 ligand-independent, constitutive activation of Flt3 kinase activity and downstream activation of signaling pathways linked to increased myeloid leukemia cell growth (recently reviewed) [273]. FLT3/ITD mutations occur in 21–38% of occidental APL patients (median >30 vs 12–25% of oriental patients), and, as noted previously, have frequently been associated with increased WBC count, the M3v phenotype, and the S-isoform of PML-RARa (11 clinical trial data recently summarized) [274]. In a minority of studies, a trend toward an association of FLT3/ITD mutations with reduced disease-free or overall survival has been observed by univariate statistical analysis [261, 263, 274–276], however, after adjustment for the associated high WBC count, no study has shown a significant reduction in postremission disease outcome (ibid; 1 study showed a significant association with increased deaths during remission induction [269, 277, 278]. In other classes of AML, quantitative assessment of ITD mutations found that only those with a high ratio of the mutant to the normal FLT3 allele were associated with poor prognosis [279]. However, no such relationship was found in two recent APL studies in which the ITD mutation was quantitatively assessed [269, 278], although in one of these studies, a long insert sequence was independently associated with an increased incidence of disease relapse [269]. In the context of the studies cited earlier, several other mutations have been tested for a possible association with APL disease outcome, including alternative FLT3 activation mutations in the tyrosine kinase domain (FLT3/RTK mutations, most affecting aspartic acid residue 835) [263, 274, 277, 278], RAS gene mutations [261], and MLL gene partial tandem duplications and Kit [263], however, the low frequency of these mutations in APL has limited statistical assessment. Finally, one study found that transcripts for the tumor-testis antigen protein PRAME (preferentially expressed antigen of melanoma) were selectively expressed in APL cells and that a low level of PRAME expression was significantly associated with reduced relapse-free survival largely independent of WBC count [280].

In summary, many studies performed to assess potential molecular prognostic markers in APL find a significant association of high presenting WBC count, hypogranular (M3V) phenotype, S-form PML-RARα, and FLT3/ITD mutations. This is consistent with the proposal that at least a subgroup of patients with these characteristics have a disease variant that significantly differs from the predominant hypergranular disease, perhaps related to different levels of initial hematopoietic progenitor cell transformation [211]. In the context of the ATRA-chemotherapy clinical trials in which this data was developed, a high WBC count clearly takes precedence over the other three elements as a prognostic indicator, although some combined features may make some additional contribution [269, 270]. In at least three recent clinical trials, however, in which frontline arsenic trioxide (ATO) was added to ATRA/chemotherapy, neither a high WBC count nor any of the previously described molecular features of PML-RARα or FLT3 were differentially related to clinical outcome [278, 281–283]. These observations suggest that in the future an alternative set of prognostic indicators will be required to identify the minor fraction of patients (probably <5%), who remain at risk of relapse after the addition of ATO to ATRA/chemotherapy-based treatment.

A third clinical application of molecular biology in APL has been molecular monitoring of subclinical disease after achieving clinical remission (referred to as minimal residual disease; MRD), using the PML-RARα gene transcript as a disease-specific marker. Two RT-PCR methodologies have been applied: (1) a conventional procedure (cRT-PCR) that provides a positive vs negative read-out determined by the presence or absence of stained gel electrophoretic bands of the DNA product derived by terminal PCR amplification of complementary DNA (cDNA) after conversion from RNA by reverse transcriptase [284]; and (2) quantitative PCR (RQ-PCR) that provides a variable read-out depending on the number of PCR cycles that are required to detect the activation of a fluorescent dye indicating the initiation of cDNA amplification (the threshold cycle or C_T), which is dependent on the concentration of the PML-RARα transcript in total cellular RNA [285, 286]. In order to assure proper primer placement for PCR amplification, both of these methods require accurate assignment of the PML-RARα isoform type at the time of diagnosis when the fusion gene is abundantly present (Fig. 21.4a, b). cRT-PCR has a detection sensitivity between 1 in 10^3 and 10^4 (determined by the ability to detect PML-RARα transcript in a serially diluted RNA from a 100% APL cell source, usually an APL cell line), while RQ-PCR sensitivity can be up to ten times more sensitive [287, 288]. A major advantage of RQ-PCR, which has now largely supplanted cRT-PCR for MRD monitoring, is that it can be more

accurately standardized. Importantly, this includes adjustment for RNA quality and efficiency of conversion to cDNA by normalization of the PML-RARα copies (calculated by comparison of the sample C_T to those for a plasmid standard curve with known copy numbers) to a constantly expressed housekeeping gene: thus, read-out is expressed as the normalized copy number (NCN) or normalized quotient (NQ).

Important lessons from the application of cRT-PCR to ATRA-chemotherapy clinical trials have included: persistence of MRD detection in the immediate postclinical remission induction period is not an indicator of long-term adverse outcome and is likely due to the slow clearance of terminally differentiated APL cells; detection of MRD after finishing consolidation therapy (molecular persistence) is a virtually certain prognostic indicator for subsequent clinical relapse if a repeat sample is also positive; and, the majority of eventual clinical relapse cases test negative at the postconsolidation checkpoint so that continued monitoring at relatively frequent intervals for 2–3 years is needed to identify most cases destined to clinically relapse [289, 290]. The clinical importance of MRD monitoring was supported by a report that the long-term outcome of patients initiated on salvage therapy based on the detection of MRD (molecular relapse) was superior to that of a historical control group in which salvage therapy was only initiated after clinical relapse [291]. This result was confirmed under internally controlled trial setting [292], and molecular relapse or persistence is now considered the equivalent of clinical relapse in calculating the relapse rate in clinical trial studies [257].

The application of RQ-PCR has confirmed the essential cRT-PCR MRD monitoring results with more accurate and refined criteria. Further, the quantitative NCN values have allowed an assessment of the kinetics of subclinical residual disease, e.g., not only whether a confirmatory, repeat test after a suspicious result is positive or negative but whether it is increasing or decreasing. From several RQ-PCR studies, it is apparent that low levels of MRD may remain detectable and yet not necessarily be a harbinger of inevitable clinical relapse, especially in the early postconsolidation treatment phase [272, 283, 287, 293, 294]. In these studies, it has been possible to define criteria—either threshold NCN values with a very high risk of subsequent clinical relapse [272, 287, 293] or compelling evidence of increasing MRD levels in serially collected samples [294]—that mandate immediate salvage therapy. In practice, prospective (rather than retrospective) RQ-PCR MRD monitoring with criteria for treatment change during the subclinical phase has only been realized in two clinical trial reports [283, 294]. In the first report, the principal clinical trial objective was to identify patients who were in subclinical relapse after ATRA-chemotherapy treatment in order to initiate early ATO salvage therapy [294]. Notable results of this study were: the clinical relapse rate after 3-years follow-up was reduced to 5% compared to 12% in an earlier similar clinical trial with no prospective MRD monitoring; there was no incidence of the differentiation syndrome when ATO salvage therapy was administered with subclinical disease which was sometimes present and problematic after hematological relapse; and MRD monitoring of bone marrow was more effective than peripheral blood. The latter is confirmation of earlier indications of this difference in studies with very limited case numbers [287, 293], although similar levels of MRD have been reported in simultaneously obtained bone marrow and blood samples, possibly related to disproportionate testing of early treatment samples [272, 287, 294]. Further, continued MRD monitoring during and after ATO salvage therapy was effective in detecting patients in incipient second relapse and in evaluating patients for transplant candidacy while the disease was still subclinical [294]. In the second report, intensive MRD monitoring was employed as a precautionary measure in an exploratory Phase II clinical trial in which frontline ATO therapy was administered with potentially insufficient, reduced-intensity chemotherapy in combination with ATRA [283]. However, among 37 patients (including 12 with high WBC counts) who completed consolidation therapy, there was only 1 relapse confined to the central nervous system and no hematological relapses (2.7% incidence of relapse with 2.7 years follow-up). Concordantly, after the completion of consolidation therapy, there were no RQ-PCR assays in bone marrow or peripheral blood that exceeded a high-risk NCN/NQ value ($>10^{-5}$; risk values defined in [272, 295]), a series of intermediate-risk values ($>10^{-6}$ but $<10^{-5}$; predominantly in blood) in the CNS relapse case, and only three assays (out of hundreds) that transiently exceeded an intermediate-risk NCN value in the remaining cases [283]. This study and other recent studies indicating that the relapse rate in APL is likely to be <5% after the addition of frontline ATO to ATRA chemotherapy even in high-risk, high WBC count patients [281, 282] bring into question the cost-to-benefit ratio of continuing to perform MRD monitoring in APL patients with primary disease. Consideration of this issue is sharpened by a recent study using mathematical modeling which concluded that to be highly effective in prospectively detecting MRD in time to implement preclinical relapse salvage therapy in APL, RQ-PCR testing would need to be performed on bone marrow samples every 2 months [296]. Thus, in the future it may be reasonable to limit MRD monitoring by RQ-PCR in primary disease to clinical trial settings in which the predicted incidence of relapse has not been established to be very low, e.g., in exploratory trials that attempt to further reduce exposure to cytotoxic chemotherapy. On the other hand, intensive application of this now highly validated method after the occurrence of increasingly rare first relapse seems indicated, since the long-term outcome of salvage therapy is less certain and molecular monitoring will likely be useful in making therapeutic adjustments to avoid secondary clinical relapses.

Clinical Features

APL accounts for about 10% of adult AML and the incidence appears to be approximately constant with respect to age [297], an observation not previously reported for any other neoplasm. The incidence in children is usually reported to be lower [298], although the incidence of APL in children diagnosed with AML and living in Italy seems to be about twice that of children living in Germany or the United States [299–301].

When Latinos develop AML they may be significantly more likely to develop APL than another AML subtype [302–305], and an investigation of the Eastern Cooperative Oncology Group database for leukemia studies E2491 and E3489 revealed that more APL patients are Latinos than are other AML patients ($P = 0.005$) [306]. Others have reported that blacks have a lower likelihood of APL than Hispanics, non-Hispanic whites, and Asians [307].

There is no difference in the incidence between the sexes [308] and the median age of patients with APL appears to be about 15 years younger than that of patients with other forms of AML (43 years vs. 59 years, $P < 0.00001$) [309]. Curiously, increasing body mass index was strongly associated with a diagnosis of APL among patients with AML ($P = 0.0003$) in one study [309] and associated with the differentiation syndrome (discussed in text to come) in another [310]. Some epidemiological studies have implicated exposure to electromagnetic fields [308] and radon exposure [308] in the etiology of APL. Therapy-related APL has been increasingly recognized [83, 311–313]. Exposure to drugs that target topoisomerase II may be especially likely to result in treatment-related APL [81]. An association with exposure to mitoxantrone for, among other diseases, multiple sclerosis, has been well established [82, 314, 315], and it has been suggested that gefitinib therapy for lung cancer may also result in secondary APL [316].

The most significant clinical feature of APL is a hemorrhagic diathesis manifested by ecchymoses, intracranial hemorrhage, or gastrointestinal bleeding, some evidence of which is present at diagnosis in the majority of patients [317, 318]. Sudden blindness due to sinus vein thrombosis has been reported [319] and other large vessel thromboses have been reported as well [320], although hemorrhage is much more common than thrombosis in APL. The most frequently documented laboratory evidence of the bleeding diathesis includes hypofibrinogenemia, increased fibrin degradation products in the serum, a prolonged prothrombin time, and, of course, thrombocytopenia [317, 318, 321]. The potential for bleeding is exacerbated by cytotoxic chemotherapy with consequent thrombocytopenia [318, 322]. The diagnosis and treatment of this important life-threatening manifestation of APL is fully discussed below.

The diagnostic hallmark of APL is the balanced cytogenetic translocation t(15;17)(q22;q11.2–12) [323] that has been discussed in detail above. However, approximately one-third of patients have additional cytogenetic abnormalities, most frequently trisomy 8 [324–326]. There may be a deleterious effect of additional cytogenetic abnormalities on the course of APL in general although the data available are conflicting. In a study of 47 APL patients, 17 with additional cytogenetic aberrations, Schoch et al. [327] found no influence of such abnormalities on prognosis. Some of their patients were treated with ATRA, others with chemotherapy. In a larger study of 161 patients treated with chemotherapy alone, Slack et al. [328] found that secondary cytogenetic changes were associated with significantly longer CR duration and event-free survival after treatment with anthracycline and cytarabine. They concluded that additional cytogenetic changes do not impair prognosis of patients with APL treated with chemotherapy. Pantic et al. [329] studied 43 APL patients treated with ATRA alone and found additional cytogenetic changes in 33%. The CR and early death rates were significantly different between those patients with and without additional abnormalities (36% vs. 76% $p = .0148$; 24 vs. 64%, $p = 0.0141$, respectively). They concluded that patients treated with ATRA who have additional cytogenetic abnormalities have a more aggressive disease than those with only t(15;17). We found this also to be true, and also that patients treated with ATRA with or without chemotherapy who had t(15;17) alone had a significantly better overall survival than did patients with additional cytogenetic abnormalities [330]. We concluded from a study of 140 patients that those with APL and t(15;17) alone were significantly more sensitive to ATRA than are patients with t(15;17) and additional cytogenetic abnormalities. Others have reported similar results in patients treated with ATRA and anthracyclines together [326] and concluded that additional cytogenetic changes may render patients less sensitive to ATRA, as suggested by the data of Pantic et al. [329] and Wiernik et al [330]. Consistent with these observations, Xu et al. [331] after a study of 284 patients with APL recently reported that relapse-free and overall survival are significantly poorer in patients with complex karyotypes compared with patients with only t(15;17). However, De Botton et al. [325] studied 292 patients treated with ATRA and chemotherapy. Additional cytogenetic abnormalities were present in 26% of cases and there was no difference in outcome between patients with and without additional cytogenetic aberrations. At present, because of conflicting data, there is no clear indication based on additional cytogenetic changes to alter what has become conventional therapy with ATRA and anthracycline-based chemotherapy [331] (discussed below). Secondary clonal cytogenetic abnormalities frequently appear in APL patients after treatment, but seem to be of no clinical importance [270, 332, 333]. Rarely, APL patients present with t(11;17) [138] as the only cytogenetic abnormality, and such patients are usually refractory to

ATRA treatment. Jansen et al. [334] induced a complete molecular remission in such a patient with the combination of ATRA plus G-CSF after demonstrating the efficacy of that regimen in vitro.

The immunophenotype of APL cells is unique among the myeloid leukemias, since they are characteristically HLA-DR-negative, CD34-negative, p-glycoprotein-negative, and CD33+ although the M3v (microgranular variant) subtype may be positive for either CD34, p-glycoprotein, or both (see Chap. 17). In addition, certain T-cell antigens, such as CD2, are frequently expressed by the FAB-M3v type of APL [318, 334, 335]. Rarely, APL cells express CD56, a neural-cell adhesion molecule. Such patients may have a poor prognosis for complete response and response duration [336].

The morphology of the leukemic cells in APL is unique and discussed in Chap. 16. Briefly, three morphological subtypes have been described. In the hypergranular type, which is the most common form of the disease, the cells appear to be abnormal promyelocytes with abundant cytoplasmic granulation that stains purple or pink with Wright's or similar stains. Auer rods are common, usually multiple, and frequently appear in bundles (haystacks) [337]. The nucleus is bilobed, folded, or reniform, suggestive of the nucleus of a monocyte [318, 337]. In the microgranular type [337], which accounts for approximately 25% of all APL cases [264, 338–340], cytoplasmic granules are difficult to see with the light microscope and Auer rods are rare. Occasional typical M3 cells are seen in the peripheral blood and are more numerous in the bone marrow. The nucleus in M3v appears similar to that of M3. M3v cells are much more commonly CD2+ than are M3 cells [335, 339] and the t(15;17) abnormality is almost always present [335, 339, 341]. A third, rare form of APL has been designated as the hyperbasophilic microgranular form [337, 342–344], by some authors. The cytoplasmic granules are intensely basophilic and prominent cytoplasmic budding is often evident in this as well as is in microgranular type. The morphology of those latter two types is reminiscent of that of micromegakaryocytes [321, 341]. Cytogenetic and immunophenotypic characteristics are typical for APL, except that in the hyperbasophilic microgranular type additional cytogenetic abnormalities such as 12p13 [344, 345], which has been described in AML with basophilic differentiation [338] may be found. At least one hyperbasophilic microgarnular patient developed hyperhistaminemia after treatment, presumably secondary to release of histamine from the basophilic granules of killed cells [346]. Both APL variants seem to have more severe bleeding at diagnosis than typical APL patients [264, 338, 342] despite higher platelet counts [339], and both are much more common in non-Whites [264, 318, 339–341, 347–349] and in females [339, 341, 343]. The determination of APL type must be made prior to therapy since arsenic trioxide therapy may induce basophilic differentiation of APL cells [350].

Histochemically, M3 cells are strongly peroxidase- and Sudan black-positive and, on occasion, strong α-naphthylacetate esterase activity sensitive to sodium fluoride may be demonstrated [337, 351–353], similar to that often observed in FAB-M4 or M5 AML. This finding is not observed in normal promyelocytes. Microgranular APL cells are less frequently peroxidase or esterase positive than typical APL cells [339, 340]. Microgranular APL may have had a poorer response to treatment and a poorer prognosis than typical APL with chemotherapy alone in the past [339], but this is no longer true when treatment includes ATRA [264].

Typically, patients with APL present with lower white blood cell counts (WBC) than do other patients with AML and counts of less than 1000 cells/μL are common [318]. Patients with microgranular APL generally present with higher WBC and leukocytosis is frequent [298, 334, 343]. Anemia is common at presentation [318] and may be severe in bleeding patients. Organomegaly, lymphadenopathy, and central nervous system (CNS) leukemia are rare in APL in the United States [298, 318], but may be more common in other countries for unknown reasons [343].

Approximately 3–5% of APL patients relapse at extramedullary sites. In the ATRA era, central nervous system (CNS) relapse appears be more common than in the pre-ATRA era and is more frequent among patients who present with a high white blood cell count [354–358]. An initial serum lactate dehydrogenase level of >3000 IU/L was recently found to be more strongly associated with CNS relapse than is an initial WBC of >10,000/μL [359]. Other factors associated with a higher incidence of CNS relapse include elderly patients, and CNS hemorrhage during induction therapy. CNS relapse is the most common form of extramedullary disease in APL [360] and usually occurs within 10 months of diagnosis. It may also rarely be diagnosed at initial presentation [361]. Other sites of extramedullary relapse include skin infiltration, [322, 349], and bone [362] after treatment with chemotherapy or ATRA [363–365], particularly among those patients who present with high a WBC. Rarely, isolated extramedullary relapse may occur after more than a decade of complete remission [362].

Most patients with APL do not have an antecedent hematologic or neoplastic disease. However, therapy-related APL with the typical t(15;17) has been reported after treatment for other neoplasms, including breast cancer, prostate cancer, non-Hodgkin's lymphoma [366], papillary thyroid carcinoma [367], and other neoplasms. In fact, most reported cases of therapy-related APL occurred in patients previously treated for breast cancer [367, 368]. This is curious because BRCA1 is located on chromosome 17 near the breakpoint involved in the formation of t(15;17) [369]. Wei et al [370] reported that ATRA inhibits a key regulator of oncogenic signaling pathways, the unique isomerase Pin1 in APL and breast cancer cells. This observation suggests some common

pathways in the etiology of both diseases.The incidence of therapy-related APL may be increasing [371]. It occurs primarily in middle-aged adults with a peak incidence at 2 years post treatment for the initial neoplasm and is more common in women [371]. Topoisomerase II inhibitors, radiation, and mitoxantrone are the most common treatments prior to the development of APL. The complete response rate and prognosis after treatment of treatment-related APL is similar to that of de novo APL [371, 372]. Prostate cancer is being recognized more frequently recently as a tumor associated with an increasing incidence of treatment-related APL [372]. In addition, karyotypically confirmed promyelocytic blast crisis of chronic myelogenous leukemia (CML) has been occasionally reported [373–376], and promyelocytic blast crisis of CML with cytogenetic abnormalities other than t(15;17), but involving chromosome 17 has been documented [377]. In fact, such a patient was the first patient with leukemia in the United States to respond to ATRA [369]. Rarely, patients with APL relapse with a cytogenetically different AML or myelodysplasia following treatment of the original leukemia [378–382]. Most such patients to date have received prior anthracycline therapy.

On rare occasion APL may be diagnosed upon relapse of other types of acute myeloid leukemia (AML) [383], and in other rare instances APL may relapse as another type of AML or myelodysplastic syndrome [384]. APL has even been reported to develop in donor cells after an allogeneic stem cell transplant [385].

Three risk groups of patients with APL have been generally recognized: low-risk, intermediate-risk, and high-risk. Low-risk patients are those with an initial WBC <10,000/μL and a platelet count >40/000/μL, intermediate risk patients are those with a WBC <10,000/μL and a platelet count <40,000/μL; high-risk patients are those with a WBC >10,000/μL irrespective of platelet count. Low-risk and intermediate-risk patients have classically been considered together for treatment purposes but therapy is usually intensified for the 10–15% of patients that are at high-risk (see below) since they are at higher risk for relapse, hemorrhage [386] and death (but usually not for lower complete response to induction therapy). Elderly patients often fall into the high-risk category [387]. Leukapheresis does not improve the prognosis for patients with hyperleukocytosis [388].

More recently, additional factors associated with therapeutic response have been identified. Patients whose leukemic blasts are CD34+, CD56+ or CD2+ are at higher risk of relapse. These antigen expressions are often associated with leukocytosis [386]. Patients with the short PML/RARα isoform as well as those with FLT3-ITD mutations (also associated with leukocytosis) are at increased risk for relapse as well in some studies [386, 389], and FLT3-ITD mutations were associated with impaired survival after treatment with ATRA and chemotherapy in one study and event-free survival in another [390]. However, importantly, FLT3-ITD mutations had no effect on outcome after treatment with ATRA plus arsenic trioxide [390]. Another study identified by multivariate analysis that the quantity of RARα transcripts in blood prior to induction therapy as the sole independent prognostic factor for relapse. At 5 years after treatment patients with >209.6 PML-RARα/ng had a cumulative incidence of relapse of >50% compared with 7.5% for those with less molecular burden of transcript [391]. Low transcript levels of KMT2E (MLL5), a gene involved in the positive control of genes involved in hematopoiesis, results in lower remission rate and shorter overall survival in patients with APL treated with ATRA and an anthracycline [392], and WT1 expression was shown to be an independent prognostic factor for overall survival of complete responders to induction therapy [393].

The TP73 gene transcript is translated into an active TAp73) and inactive (ΔNp73) isoforms. Higher inactive form/active form RNA ratios are associated with a higher risk of relapse and poorer survival of patients treated with ATRA plus anthracycline [394]. Lastly, data suggesting that ETV6 rearrangement may be an independent unfavorable prognostic factors for overall survival in patients with APL [395].

Most of the prognostic factors described above have been identified in patients treated with ATRA and anthracycline chemotherapy. Since arsenic trioxide is playing an increasingly important role in the treatment of APL it will be important to verify which, if any, of these factors still are reliable prognostic indicators that can be used to stratify patients among various therapeutic approaches. Recently, Lou et al. [396] addressed this question in 184 patients treated with arsenic trioxide-base therapy. They found no significant association between 3-year relapse-free survival and initial WBC count, FLT3-ITD status or type of PML-RARα isoform. Only CD56 in their study retained prognostic value with respect to relapse-free survival. These data suggest that arsenic trioxide-based therapy is superior to ATRA plus anthracycline treatment and that some previously important prognostic factors are no longer relevant. Further studies of this kind are needed. It may be necessary in the near future to revise commonly used risk assessment schemes for patients with APL.

The primary cause of treatment failure for patients with APL is death caused by the coagulopathy characteristic of this disease [397]. The early death rate is approximately 10% in patients <50 years of age and 30% in older patients. It is higher in patients not enrolled in clinical trials than in patients enrolled in such trials [398, 399]. Death from this cause may even occur before treatment is begun. Early death rates may be higher in uninsured patients and among minority populations [400]. Therefore, it is imperative to remove the societal obstacles to early, competent care for these subpopulations.

Treatment of APL

APL patients must be prepared for induction therapy as described for other FAB types in Chap. 22 whenever possible, but time is of the essence in APL more so than in other FAB types in that the diagnosis of APL is a medical emergency that requires immediate intervention. Once the diagnosis is suspected and while it is being confirmed by polymerase chain reaction or by immunofluorescence staining with an antipromyelocytic leukemia antibody [401], aggressive supportive care measures with blood product support should be instituted, and ATRA should be started. If the diagnosis of APL is not confirmed, ATRA should be discontinued. Concurrent with initiation of induction therapy, the hemorrhagic diathesis, which clinically or subclinically is present in virtually every patient, must be brought under control since such therapy may exacerbate the bleeding problem, particularly in patients with leukocytosis. Specific recommendations for controlling hemorrhage are given next [402, 403].

Induction Therapy

The response of APL to appropriate induction therapy is unique among the acute leukemias. As initially pointed out by Kantarjian et al. [9] and by Daly et al. [317], hematologic recovery from induction chemotherapy in at least 85% of patients is accompanied by an increase rather than a decrease of promyelocytes in the bone marrow, which spontaneously mature over several or more weeks. Postinduction therapy bone marrow specimens with increased promyelocytes have frequently been erroneously interpreted as indicative of treatment failure and retreatment has led to the death of patients. Virtually no patients who survive induction therapy will require a second course because primary resistance of leukemic promyelocytes to commonly used agents, such as ATRA and arsenic trioxide is extremely rare. Resistance to ATRA when it occurs is associated with mutations in the RARα moiety of PML-RARα and resistance to arsenic trioxide is associated with mutations in the PML moiety. Both mutation types interfere with degradation of PML-RARα, the gene that drives the disease [404]. Both agents synergistically degrade mutated NPM1 genes which results in apoptosis [405].

History of Specific Induction Chemotherapy

Anthracyclines have been the cornerstone of chemotherapy for APL since the landmark observation of Bernard et al. that daunorubicin induced a high percentage of complete responses in this leukemia [5] and that CRs were of unusually long duration, compared with those in other morphologic subtypes of AML. Since that discovery, the most common treatment reported for APL has been the standard daunorubicin and cytarabine regimen originally described by Yates and colleagues [411] and recommended by them for all FAB types. There is, however, little evidence that cytarabine is actually necessary as an induction agent in APL [407, 408]. At least 350 patients with APL treated with daunorubicin alone can be gleaned from the literature, with an overall CR rate >70% [317, 342, 345, 409–413]. This result compares very favorably with the 67% CR rate published from 1990 to 2010 for 537 patients induced with daunorubicin and cytarabine with or without other chemotherapy [318, 406–421].

In a trial of idarubicin and cytarabine compared with historical controls treated with daunorubicin and cytarabine [422, 423], the former yielded significantly greater disease-free and overall survival compared with the latter. This result may be valid, since the same advantage for idarubicin and cytarabine over standard therapy has been reported for AML in general [424, 425], and others [426] have reported an unusually high complete response rate with idarubicin alone in APL. Newer strategies have included lower doses of idarubicin in children combined with ATRA [427]. The Italian GIMEMA group and Spanish PETHEMA group have reported high complete response rates with ATRA and anthracycline, and the combination became a standard induction regimen for APL [266, 408, 428–434].

ATRA Induction Therapy

Orally administered ATRA induces complete, albeit relatively brief remissions in the vast majority of patients with APL. Unfortunately, relapse usually occurs within months if no postremission therapy is given. Complete remission with ATRA is accomplished by induction of differentiation and maturation of leukemic cells, and not by a cytotoxic mechanism [431]. Clinical evidence of this fact includes the occasional observation of mature neutrophils in responding patients that contain Auer rods, suggesting that these neutrophils are matured leukemic cells [432].

Pharmacology of ATRA

Orally administered ATRA, 45 mg/m^2, results in a peak plasma concentration in 1–2 h [433] and is rapidly eliminated from humans, with a terminal half-life of approximately 45 min after an initial dose [434]. Following long-term daily administration of the agent, plasma concentrations of ATRA decrease significantly over time [218]. The only known metabolite is 4-oxo-all-*trans* retinoic acid, which is

found in plasma and urine, but accounts for only about 10% of administered ATRA [433, 435]. ATRA does not enter the cerebrospinal fluid [433]. The mean area under the curve for plasma drug concentration of ATRA varies considerably from patient to patient [433, 434]. It was suggested that relapse on continuous administration of ATRA may be due to the progressive reduction of plasma concentration described previously to levels below those that effect leukemic cell differentiation, since leukemic cells from patients who relapsed on ATRA usually continued to be sensitive to the agent in vitro at ATRA concentrations that resulted in differentiation initially. However, it is now known that marked decreases in plasma concentration occur within days of initial administration, long before relapse occurs in virtually all patients [433]. Induction of accelerated catabolism by a cytochrome P-450-like enzyme system has been suggested as a mechanism for these peculiar aspects of ATRA catabolism [433]. This suggestion has merit, since inhibitors of oxidation by cytochrome P-450 enzymes such as ketoconazole, fluconazole [436], and liarozole [437] significantly increase plasma concentrations of ATRA and decrease concentrations of the oxo-metabolite when administered with ATRA. A recently offered possible alternative explanation for the accelerated ATRA catabolism observed after its continuous administration is that ATRA administration appears to result in increased levels of plasma lipid hydroperoxides, which accelerate the oxidative catabolism of ATRA in human microsomes in vitro [438]. And, of course, it is quite possible that ATRA blood levels correlate poorly with ATRA activity because they are irrelevant. Intracellular ATRA concentrations are much more determinant of ATRA activity [439].

Clinical Results with ATRA

Huang et al. [12] first reported on the clinical usefulness of ATRA in APL in 1988. They treated 16 previously untreated and eight previously treated patients with oral ATRA, 45–100 mg/m^2/day. All patients achieved a hematologic and clinical CR without developing bone marrow hypoplasia. Eight patients experienced early relapses within 5 months while still receiving ATRA, but the others remained in remission at the time of their publication for as long as 11+ months. This landmark observation that ATRA could induce remissions in the vast majority of patients with APL presumably by differentiation induction attracted little attention initially in the United States, but French collaborators of the Chinese investigators immediately recognized the importance of this observation and initiated their own trials, which proved to be confirmatory [440].

Chen et al. [215] observed that patients who were induced into remission with ATRA and then maintained with conventional chemotherapy could usually be successfully reinduced into second remission with ATRA after relapse, whereas patients who received both ATRA and chemotherapy as postremission treatment usually could not be induced into second remission with ATRA alone. Based on these observations, they suggested that ATRA should be discontinued upon the achievement of CR.

Subsequent studies by a large number of investigators demonstrated that, while the vast majority of patients with APL achieved CR with ATRA, some patients, perhaps 10–20%, did not respond well, primarily due to early death. In addition, a serious problem with ATRA therapy, initially termed the retinoic acid syndrome [441, 442] but now referred to as the APL differentiation syndrome, became evident (see below). This occasionally fatal, rapidly developing pulmonary distress syndrome is not related to pulmonary leukostasis, but is often but not always associated with a rapidly rising peripheral WBC count consisting of predominantly maturing cells. In initial studies, the differentiation syndrome developed in as many as one-quarter of patients within days or weeks of starting ATRA therapy. This observation, and those of Chen et al. [215] discussed earlier, led Fenaux et al. [443] to combine ATRA induction therapy with postremission chemotherapy on a flexible schedule. Their plan was to begin treatment with ATRA and switch to anthracycline-based chemotherapy after remission was achieved unless a rising WBC occurred with ATRA, a dangerous harbinger of the differentiation syndrome, in which case chemotherapy was administered early along with ATRA until CR was documented, after which chemotherapy was continued. A CR in 96% of patients was achieved with this approach in a pilot study of 26 patients and the actuarial disease-free survival of 87% at 18 months was significantly better than the 59% rate observed in their previous chemotherapy-alone study [443]. This pilot study led to a larger multi-institutional study in which 101 patients under the age of 65 years were randomized to receive daunorubicin and cytarabine alone, or those drugs preceded by ATRA treatment [444]. Both groups received two courses of daunorubicin and cytarabine as consolidation therapy after complete remission was achieved. The three-drug induction regimen yielded a 91% CR rate and a 9% death during induction rate, compared with an 81% complete response rate and an 8% death during induction rate for the two-drug regimen. Neither of the differences is significant. However, the estimated 4-year event-free survival in the ATRA plus chemotherapy induction group was 63% compared with 17% in the chemotherapy-alone group, which is a highly statistically significantly different outcome. Overall survival at 4 years for the combined modality and chemotherapy-alone groups was 76% and 49%, respectively, which is also a highly significant difference [445]. Therefore, although the addition of ATRA to standard induction chemotherapy did not result in a significantly higher CR rate or reduced early death rate, disease-free survival of complete responders was greatly enhanced.

In addition, ATRA therapy rapidly resolved the coagulopathy in most patients, an observation subsequently confirmed by others [446]. This approach to APL treatment was validated by Kanamaru et al. [447], who obtained results virtually identical to those of Fenaux et al. [444]. In that study [447], approximately 89% of 110 patients achieved CR with ATRA alone or ATRA and early chemotherapy and 6.3% developed the differentiation syndrome. With a median follow-up of 21 months, 81% of the complete responders were projected to be disease free at 23 months, which was significantly greater than the disease-free survival rate observed in a prior chemotherapy-alone study. Unlike the study reported by Fenaux et al. [445], the Japanese study demonstrated a significantly lower early mortality rate than observed in the chemotherapy-alone historical control [447]. Burnett et al. [267] demonstrated that prolonged ATRA administration starting simultaneously with chemotherapy and continuing throughout the induction period until complete response is diagnosed gave superior results compared with a short course of ATRA prior to chemotherapy. Patients in the former group had a significantly higher CR rate and fewer induction deaths, as well as superior survival at 4 years, compared with those treated with a short course of ATRA.

Liposomal ATRA given intravenously may be more active than orally administered ATRA [222, 448, 449]. When administered every other day at a dose of 90 mg/m^2, blood levels are maintained, rather than observed to decline as is the case with orally administered ATRA. Results are similar with the two formulations, but liposomal ATRA may be more likely to yield a molecular remission as determined by PCR than is oral ATRA [448]. However, liposome-encapsulated ATRA is no longer available, but a new preparation of ATRA loaded in cholesteryl butyrate solid lipid nanoparticles appears to be superior to ATRA alone against APL cell lines and may eventually come to clinical trial [450].

ATRA in conjunction with anthracycline chemotherapy or arsenic trioxide is superior to either approach alone for initial treatment of APL. The combined approach may not reduce the early death rate associated with APL therapy, however. Furthermore, CR rates with chemotherapy, ATRA, or a sequential combination of both appear to be quite similar. The major advantage for combined modality treatment with ATRA and chemotherapy is the significantly greater disease-free and overall survival achieved with the combination in virtually all controlled trials [451]. Yet another benefit appears to be a reduction in the incidence of the differentiation syndrome with concurrent therapy [452]. In addition, patients treated with ATRA prior to chemotherapy may have more rapid recovery of the peripheral granulocyte count compared with patients treated with chemotherapy alone [453].

More than 90% of newly diagnosed patients with APL initially treated with ATRA and idarubicin achieve a complete remission with that therapy. Almost all induction failures are due to death during induction from hemorrhage, infection, or differentiation syndrome which result in 5%, 2–3%, and 1–2% deaths of all treated patients, respectively. Elevated serum creatinine, increased peripheral blast count, and presence of coagulopathy correlate with an increased incidence of death from hemorrhage. Age >60 years, male sex, and fever at presentation correlate with an increased likelihood of death from infection, and poor performance score as well as hypoalbuminemia correlates with an increased likelihood of developing a fatal differentiation syndrome [454].

Whether other retinoids [221, 455–459] are superior to ATRA in the treatment of APL needs to be determined as well. Observations on the potentiation of megakaryocytopoiesis by ATRA [460, 461] and on the inhibition of marrow angiogenesis by ATRA [462] require further study, as does the observation that imatinib [463] and statins [464, 465] may potentiate ATRA activity against APL.

Retinoic Acid Toxicity

Differentiation Syndrome

The most serious toxicity associated with ATRA therapy is the APL differentiation syndrome [466] which occurs in 25% or more of patients treated with ATRA alone [467], or in combination with anthracycline chemotherapy [468]. Differentiation syndrome may also be induced by arsenic trioxide [469]. Half the patients who develop the syndrome have a severe form which can be fatal, and the others have a moderate form from which recovery is the rule [468]. Severe, life-threatening differentiation syndrome usually occurs in the first 2 weeks of treatment, while a milder form may occur later [470]. Fever and respiratory distress with or without pulmonary infiltrates on chest radiograph are the hallmarks of the syndrome. Weight gain, pedal edema, pleural and pericardial effusion, and hypotension may also occur. A bimodal incidence of the syndrome is reported with peaks occurring in the first and third weeks after the initiation of ATRA therapy [471]. Rarely, a patient may develop the syndrome during both peak incidence periods [471]. Autopsy reveals massive pulmonary parenchymal tissue infiltration with maturing myeloid cells [467]. A white blood cell count >5000/μL and a serum creatinine concentration above normal correlate with an increased risk for severe differentiation syndrome [472]. In most, but not all patients the syndrome is preceded by a rapidly rising white blood cell count. ATRA must be discontinued at once when the manifestations of the syndrome are severe and dexamethasone, 10 mg intravenously every 12 h should be administered until complete clinical resolution of the syndrome is obtained, usually in several days. Some evidence suggests that prophylactic

dexamethasone can reduce the incidence of the syndrome [472]. If the manifestations are mild, ATRA can be continued with the institution of dexamethasone. Most patients so treated will survive and, once the syndrome has resolved, ATRA therapy can usually be safely reinstituted, but it is recommended that resumption of ATRA be carried out under the coverage of steroids [467]. The syndrome rarely, if ever, occurs in patients receiving ATRA as postremission therapy [467]. Curiously, there are no data on hydroxyurea as treatment for this syndrome.

The early (concurrent) administration of chemotherapy with ATRA for induction appears to have a benefit with respect to reduction in the incidence of this syndrome [468]. Diffuse pulmonary hemorrhage may mimic the syndrome or may be a manifestation of it [473, 474].

The pathogenesis of the ATRA syndrome is not entirely known. Expression of CD13 by APL cells obtained at diagnosis significantly correlated with the development of the ATRA syndrome in one study [473, 474], which is interesting since expression of that antigen (aminopeptidase N) has previously been associated with a poor prognosis in AML and with tumor invasive capacity in some human tumor cell lines. It has been suggested that bestatin [466], a specific inhibitor of aminopeptidase N, should be tested as a possible prophylactic agent against the ATRA syndrome. It has also been demonstrated that ATRA upregulates CD54, CD11b, and CD18 on APL cells, which facilitates adhesion of them to pulmonary microvasculature, which can be reversed in vitro by anti-CD54 and anti-CD18 antibodies [475]. Furthermore, ATRA may induce chemokine production in the lung and in APL cells which enhance migration of the leukemic cells out of the vascular system [476–478].

Other ATRA Toxicity

Other toxicities associated with ATRA therapy are usually mild and include dry mucous membranes, bone pain, headache, hypertriglyceridemia, hepatic enzyme elevation, and skin rash, which may rarely evolve into erythema nodosum [479]. Pseudotumor cerebri has been reported in approximately 2% of patients receiving ATRA. The incidence is higher in children and young adults than in older patients [480, 481]. The cause is entirely unclear, but recently thrombophilic factor dysmetabolism has been implicated [482]. Treatment in APL patients usually only requires diuretics. Sweet syndrome [483, 484] has rarely been reported after ATRA administration, presumably due to a mechanism similar to that of the ATRA syndrome. Occasionally, serious thrombotic episodes may occur with ATRA therapy even when thrombocytopenia is present [485]. ATRA-induced thrombocytosis has also been observed [486, 487].

Patients treated with ATRA and anthracycline-based chemotherapy have approximately a 2% incidence of an acute myeloid leukemia or myelodysplastic syndrome developing in a median of 4 years after completing treatment for APL. The treatment-related myeloid disorder is associated with deletions of chromosomes 5 and/or 7, or 11q23 rearrangements. Patients over the age of 35 years have a higher incidence of this complication than others (approximately 5%) [488].

Clinical Results with Arsenic Trioxide

The most important new development in the treatment of APL in recent years is the introduction of arsenic trioxide (ATO) as a therapeutic agent. The drug was used in the late 1800s in the treatment of CML with some success [489]. Shen et al. [182] reported the activity of this agent in APL 2 decades ago. They administered ATO, 10 mg daily as a continuous intravenous infusion to ten patients who relapsed after ATRA induction and chemotherapy maintenance and achieved a clinical CR in 90% [17] without significant toxicity. Soignet et al. [187] subsequently treated 12 patients with APL who had relapsed after extensive prior therapy with ATO doses ranging from 0.06 to 0.2 mg/kg/day until bone marrow remission could be documented morphologically. Eleven patients achieved a complete remission after 12–39 days of treatment and a total dose of 160–515 mg. Eight of 11 patients who initially had a positive RT-PCR assay for the PML-RARα fusion transcript tested negative during remission. Three other patients remained PCR positive and relapsed early. This is an important observation, since ATRA therapy alone rarely results in a negative test. Side effects were also reported to be minimal in this study.

Investigators at the Shanghai Institute of Hematology [490] reported on 47 relapsed and 11 newly diagnosed patients with APL treated with ATO; 8 of the newly diagnosed patients (73%) and 40 of the relapsed patients (85%) achieved a CR with a median disease-free survival of 17 months. Patients received a variety of postremission treatments and those that received postremission chemotherapy plus ATO had significantly longer remissions ($P = 0.01$). Unlike previous studies, however, serious ATO toxicity was reported in this study. Seven cases of significant hepatic toxicity, including two deaths, were observed, and in other recent studies other toxic effects of ATO such as renal failure, cardiac dysfunction, and chronic neuromuscular degeneration have been observed [491]. Furthermore, the frequent occurrence of leukocytosis (58% of patients in one study [492]) and the differentiation syndrome (31% of patients in the same study [493]) after treatment with ATO is now well documented [493].

In an effort to limit exposure to ATO, Kwong et al. [494] treated eight patients with relapsed APL with ATO, 10 mg daily dose intravenously until remission was achieved and then gave three monthly cycles of idarubicin. All patients were in molecular remission after idarubicin treatment, and six have remained so after a median follow-up of 13 months. Jing et al. [207] reported that the combination of ATO and ATRA may be more effective therapy than either drug alone. Others have reported that ATO plus GM-CSF may be more effective therapy than ATO alone [495].

Recently, there has been an evolution in the induction and consolidation therapy in newly diagnosed patients with APL (including treatment-related APL) [283] with less chemotherapy and the introduction of the combination of oral ATRA and intravenous ATO [244, 245, 281, 282, 496–498], or ATO alone [499]. Results appear to be at least comparable to those obtained with ATRA and an anthracycline, and in many comparative studies of ATRA plus arsenic trioxide compared with ATRA plus anthracycline chemotherapy results have been better with the former, especially in low- and intermediate risk patients [500–506]. The North American Leukemia Intergroup study C9710 [283] randomized 481 newly diagnosed adult APL patients to either ATRA plus daunorubicin and cytarabine followed by two courses of consolidation therapy with ATRA plus daunorubicin, or the same treatment plus two 25-day courses of ATO. After that treatment patients were randomized to receive 1 year of maintenance therapy with either ATRA alone or in combination with methotrexate and 6-mercaptopurine. A complete remission was obtained in 90% of patients with each treatment and patients were eligible for postremission therapy. Event-free survival was significantly better for patients who received ATO compared with those who did not (80% and 63%, respectively at 3 years, $P \leq 0.0001$). Overall survival was also better for patients who received ATO (86 vs. 81% at 3 years, $P = 0.059$) as was disease-free survival (90% vs. 70% at 3 years) ($P < 0.0001$). The study demonstrated a significantly better outcome for patients who received ATO in addition to standard induction and consolidation therapy. Whether postremission methotrexate and 6-mercaptopurine had any influence on results is unclear. In a more recent prospective, randomized study of 263 low or intermediate-risk patients [507], ATRA plus arsenic trioxide or ATRA plus chemotherapy was given. The complete response rate (100% vs. 97%), event-free survival (97.3% vs. 80%), cumulative incidence of relapse (1.9% vs. 13.9%) and overall survival at 50 months (99.2% vs. 92.6%) all favored the ATRA plus arsenic trioxide regimen. These studies demonstrate major activity for ATO plus ATRA in newly diagnosed patients with APL.

Results with ATRA plus ATO have led some experts to recommend that that regimen be adopted as standard of care for patients with low- or intermediate-risk disease as currently defined, and that the addition of an anthracycline to induction therapy should only be considered for high-risk patients [508]. Others have reported better quality of life for patients treated without an anthracycline [509], and that the omission of chemotherapy improves the cost- effectiveness of treatment in the USA in some studies [510].

Recently, oral preparations of arsenic have been developed and early clinical trials demonstrate that oral arsenic is as effective and no more toxic than intravenous arsenic trioxide [511–513]. These results have led to a proposal that low- and –intermediate-risk APL patients could be treated entirely as outpatients with the oral combination of ATRA and arsenic [514]. However, others have wisely cautioned that treating APL patients during induction without hospitalization could be dangerous in the first few weeks, when coagulopathy and differentiation syndrome may have sudden onset and lead to early death [515].

Toxicity of Arsenic Trioxide

Long-term complications of ATO plus ATRA treatment were reported by Zhu et al. [516] in 265 patients who received arsenic trioxide plus ATRA for APL with or without chemotherapy. They compared quality of life and a number of potential long-term problems with those of 112 age and gender matched healthy controls. Signs of chronic arsenic toxicity such as cardiovascular events, chronic renal insufficiency, diabetes and neurological dysfunction were not observed in the patients at a higher rate than in the controls. Only mild liver dysfunction (15.2%) and hepatic steatosis (42.9%) were observed more frequently in patients than controls. Acute liver dysfunction occurred in 42.9% of patients during treatment and was managed primarily by suspending ATO treatment until liver function normalized. The estimated 12-year survival rate for patients with low- and intermediate-risk APL was 87.4%, compared with the high-risk group survival rate of 77.5%. No increased incidence of second malignancies was observed in treated patients in this study. Quality of life for long-term survivors in general was impaired somewhat, with more than half of patients reporting mild to moderate weakness and 41.1% reporting memory problems. The accumulation of arsenic in hair or nails was similar in patients and controls. Liver damage has been observed in other studies of ATRA plus ATO as well [517].

Of some concern is the reported serious cardiac toxicity that can be associated with ATO treatment. Ohnishi et al. [518] treated eight patients with APL with ATO, 0.15 mg/kg administered as daily 2-h infusions for a maximum of 60 days. Five patients achieved CR. Prolonged QT intervals were observed in all patients during treatment and ventricular premature contractions occurred during 75% of treatment

courses. Four patients required treatment for unsustained ventricular tachycardia. Unnikrishnan et al. [519, 520] and Naito et al. [521] reported patients who developed torsades de pointes (a form of ventricular tachycardia that has been observed in arsenic poisoning) after treatment with ATO, 10 mg total daily dose as a continuous intravenous infusion. Westervelt et al. [522] reported three sudden deaths among ten patients with relapsed APL who received ATO, 0.1 mg/kg/day intravenously. One of the patients became asystolic and died while being continuously monitored with cardiac telemetry, and the cause of death was unknown in the other two. It seems clear from these reports that the cardiac toxicity of therapeutic doses of ATO is greater than initially appreciated, and that fact must be taken into account in future studies, which must include careful cardiac monitoring [493, 523] and correction of hypokaemia and hypomagnesemia, if present, prior to ATO treatment [524].

More data need to be accumulated before we can be confident that we know the full story of arsenic short- and long-term toxicity. For instance, ATO may delay hematopoietic recovery after autologous stem cell transplantation [525], and accumulation of arsenic in thyroid tissue may have led to at least one late thyroid carcinoma [526]. Although patients who receive ATO seem to have a lower death during induction rate in some studies, it does not appear to resolve the coagulopathy associated with APL more quickly than treatments that do not contain ATO [527].

The observation that ascorbic acid can potentiate the activity of ATO in vitro [528] deserves clinical evaluation [529] in patients with APL, and the observation that ATO inhibits hepatitis C virus RNA replication deserves further exploration as well [530].

Postremission Therapy in APL

There is no question that consolidation therapy after successful induction therapy is necessary for optimal long-term results in APL, but the need for maintenance therapy after consolidation therapy is less clear. Initial studies from the Italian cooperative oncology group GIMEMA administered three courses of chemotherapy with intermittent-dose cytarabine plus idarubicin, mitoxantrone plus etoposide, and standard-dose cytarabine plus idarubicin plus 6-thioguanine [531]. Subsequently, it became apparent that cytarabine might not be important in induction and consolidation [266, 407, 532]. The Spanish cooperative oncology group PETHEMA treated patients with three courses of chemotherapy without cytarabine with excellent results [408, 429]. However, recent studies have shown that intermediate-dose or high-dose cytarabine appears effective in patients who present with high-risk disease [533–536]. The North American Intergroup, as indicated earlier, reported a prospective randomized trial showing that two cycles of early consolidation with ATO improves disease-free, event-free, and overall survival [282].

Maintenance therapy has fallen into disfavor, in general, in subtypes of AML other than APL although some studies strongly support its use [537]. Methotrexate and 6-mercaptopurine were reported to be particularly useful maintenance agents in APL years ago by Kantarjian et al. [538]. The North American Intergroup Study [539, 540] conclusively demonstrated the value of postremission ATRA therapy in one of the first large studies using ATRA. In that study, 350 patients were randomly assigned to induction therapy with standard doses of daunorubicin and cytarabine, or ATRA. Patients who achieved complete remission received another course of the successful induction regimen followed by a course of high-dose cytarabine plus standard-dose daunorubicin and were then randomized to maintenance therapy with ATRA, 45 mg/m^2 daily orally for a year, or observation. With a median follow-up of more than 6 years, the 5-year disease-free and overall survival rates for all patients induced with ATRA were substantially better than those for patients induced with chemotherapy (64% vs. 30%, $P < 0.0001$; 69% vs. 45%, $P = 0.0001$, respectively) although complete response rates were similar (73% vs. 70%, respectively). The 5-year disease-free survival was highest, 74%, in the subgroup of patients induced with ATRA and maintained with ATRA, which compared favorably to the 55% disease-free survival rate observed in those induced with ATRA who did not receive postconsolidation ATRA. Furthermore, providing ATRA maintenance to chemotherapy-induced complete responders improved the disease-free survival rate threefold, compared with observation alone. These data strongly indicate the value of ATRA therapy during remission. That study also demonstrated a low late relapse rate. With a median of almost 12 years of follow-up, only 4.6% of patients in complete remission for more than 3 years subsequently relapsed [541]. Equally compelling data on the value of maintenance therapy in APL come from Fenaux et al. [445]. In a study of 413 patients, those investigators randomized patients in complete remission to observation, intermittent ATRA (15 days every 3 months for 2 years), 6-mercaptopurine and methotrexate for 2 years, or that therapy plus intermittent ATRA for 2 years. The relapse rate at 2 years was 25% for patients who received no ATRA during remission and approximately half that for patients who did, 27% for patients who received no chemotherapy and less than half that for patients who did. The highest relapse rate (approximately 30%) was in the group that received no maintenance therapy at all. The study, therefore, confirms the value of maintenance therapy in APL with either ATRA or 6-mercaptopurine and methotrexate, and suggests that both are effective alone, but not additive. Long-term follow-up of this trial continued to show a benefit for

maintenance therapy which significantly reduced the 10-year cumulative incidence of relapse from 42.3 to 33%, 23.4%, and 13.4% with no maintenance, maintenance with intermittent ATRA, continuous 6-mercaptopurine and methotrexate, and both treatments, respectively ($P < 0.001$). However, some trials have suggested that maintenance with neither ATRA nor low-dose chemotherapy as discussed earlier nor the combination nor intensive chemotherapy [542] is effective in improving outcome among patients who are molecularly negative after intensive anthracycline-based chemotherapy.

In one large study ATO consolidation improved event-free and disease-free survival, compared with patients who did not receive ATO consolidation, but it was not clear from that study whether arsenic trioxide was better than other consolidation approaches [282, 543]. Couutre et al concluded from a study of 105 patients in molecular remission post-consolidation therapy that maintenance therapy is not helpful if ATO is included in the consolidation regimen [544]. Leech et al. demonstrated that a 4 month ATO-based consolidation program was equally effective in high-risk and lower risk APL patients [507]. Liu et al. [545] reported on a retrospective study of 18 patients that is clearly in need of confirmation, but interesting nonetheless. They induced low-risk patients with ATRA plus ATO while high-risk patients received the same plus an anthracycline. After hematologic complete remission occurred arsenic alternating with chemotherapy was given as consolidation therapy and no maintenance therapy was employed. All patients achieved a molecular complete remission and no patients died during treatment, and no patients have relapsed with a median follow-up of 5 years. All 18 remain alive and can be considered cured [541]. Other recent studies have also suggested that maintenance therapy after consolidation therapy is unnecessary for low- and intermediate-risk patients with APL [546].

In a randomized study of 344 patients reported by Shinagawa et al. [458] results of maintenance therapy with ATRA or tamibarotene, a retinoic acid derivative also known as AM80, were compared. There was no difference in relapse-free survival for low-risk patients between the treatments, but for the 52 high-risk patients the relapse-free survival rates for ATRA and tmibarotene maintenance were 58% and 87%, respectively, which was significant. These data do not help determine whether *any* maintenance therapy is necessary for low-risk patients, but they do suggest that it is necessary for high-risk patients and that tamibarotene may be a better maintenance agent than ATRA for those patients.

It is important to monitor all patients with APL in clinical remission after treatment with molecular techniques such as real-time quantitative polymerase chain reaction to detect leukemia-specific transcripts, should they reappear. Patients who are PCR negative after treatment should be retreated if such transcripts reappear and before there is clinical hematologic or cytogenetic evidence of relapse in order to have the best opportunity of achieving a second molecular remission [294, 547, 548]. Patients who do not obtain a molecular remission after planned therapy should continue treatment with a different agent, such as ATO with or without other agents. Institution of salvage therapy at molecular relapse before hematological relapse leads to a better outcome of salvage therapy [292, 547].

Hematopoietic stem cell transplantation is not recommended for patients with APL in first molecular remission.

Recommended treatment for newly diagnosed patients with APL is detailed in Table 21.2.

Table 21.2 Recommended treatment: newly diagnosed and treatment-related APL

Induction therapy
Low-, intermediate-, and high-risk patients
ATRA[a], 45 mg/m^2/day orally in two divided doses until marrow hematologic remission
Idarubicin, I.V., 12 mg/m^2/day × 4 on alternate days
OR
ATRA[a], same oral dose and schedule until hematologic remission
ATO[b], 0.15 mg/kg IV daily until hematologic remission
Perform PCR for PML/RARα before starting therapy and when bone marrow morphologic remission is achieved. Monitor for coagulation abnormalities, arsenic toxicity, and differentiation syndrome as described in the text. Consider steroid prophylaxis for differentiation syndrome for high-risk patients.
Consolidation therapy
Low- and intermediate-risk patients
ATRA[a], orally, 45 mg/m^2/day in two divided doses X 45 days
Idarubicin, I.V. 5 mg/m^2/day, days 1–4
Idarubicin I.V. 12 mg/m^2 day 45
OR
ATO[b], I.V. 0.15 mg/kg/day 5 days/week × 4 weeks every 8 weeks for 4 cycles
ATRA[a], orally, 45 mg/m^2/day in two divided doses for 2 weeks every 4 weeks for 7 cycles
High-risk patients
ATRA[a], 45 mg/m^2/daily orally in two divided doses × 45 days
Idarubicin, 5 mg/m^2 I.V. day 1
Cytarabine, 1 g/m^2 I.V. daily, days 1–4
Idarubicin, 12 mg/m^2 I.V. day 30
Cytarabine, 150 mg/m^2 I.V. daily, days 30–33
OR
ATO[b] IV 0.15 mg/kg/day × 5 days for 10 weeks, then
ATRA[a] 45 mg/m^2 orally daily × 7 days plus
Idarubicin, 12 mg/m^2 I.V. daily × 3 days. Repeat same dose and schedule of idarubicin after counts recover
Perform PCR for PML/RARα after hematologic recovery and every 3 months for 2 years. If PCR becomes positive after being negative, repeat in 1 month. If positive again, the patient requires reinduction therapy for relapse. Consider a clinical trial. If PCR remains negative for 2 years, no further therapy is recommended.

[a]All-trans retinoic acid
[b]Arsenic trioxide

Treatment of Relapsed APL

Whether to begin treatment for relapsed APL early (molecular relapse, normal morphology) or late (morphologic relapse) is the subject of debate. No prospective, randomized trials have been reported, but an important historically controlled study suggests a major advantage for treatment of molecular relapse [547]. If the relapsed patient has had no exposure to ATO for at least 6 months reinduction therapy with ATO, 0.15 mg/kg IV daily plus ATRA, 45 mg/m^2 daily in two divided doses until marrow remission is documented should be given. A number of studies have now confirmed excellent activity of ATO, either alone or combined with ATRA, in patients with relapsed APL [248, 549–553].

Consider CNS prophylaxis with intrathecal cytarabine at this point. If the marrow is in molecular remission, consider an autologous stem cell transplant [554, 555, 557]. If the patient is not a candidate for a transplant, consider consolidation with arsenic trioxide, or a clinical trial. If a second morphologic remission is achieved, but not a second molecular remission, consider a clinical trial.

If the patient relapsed within 6 months of treatment with ATRA and/or ATO without an anthracycline, reinduction with ATRA, idarubicin and arsenic trioxide is recommended, followed by the same post-remission treatment outlined above. If the early relapsed patient did previously receive an anthracycline, only ATRA and arsenic trioxide should be given as reinduction therapy, again, followed by the above post remission recommendations. Patients who do not respond to reinduction therapy with a complete remission should be considered for a clinical trial [556].

Tamibarotene (Am-80) is a synthetic retinoid that is a more active inducer of differentiation in HL-60 and NB4 cells than is ATRA [558]. Of 24 evaluable patients with relapsed APL after ATRA-induced CR treated with Am-80, 6 mg/m^2 orally daily, 58% achieved CR [221]. Four patients relapsed within 6 months, but long-term responses (>49 months) were also observed. The same group recently updated that study but, curiously, no new patients had been entered in the trial for approximately 3 years [559]. A more recent small study of tamibarotene in relapsed patients initially successfully treated with ATRA and ATO achieved a complete molecular response in 21% of patients. Most patients relapsed with a few months and the median overall survival after treatment was only 9.5 months [459]. The drug should be studied in combination with idarubicin for patients relapsing after ATRA and ATO.

Liu et al. treated 31 patients with refractory APL with compound realgar natural indigo tablets (an oral arsenic compound based on Chinese herbal medicine) and chemotherapy. The complete response rate was 90.3% and the median response duration was over 42 months. The relapse rate was <10%, with 43 months median follow-up and the treatment was apparently very well tolerated. Patients received a variety of chemotherapy regimens and it is difficult to know exactly what role the oral arsenic compound played in the results. However, these results are excellent. Hopefully they will soon be explored by others.

Chendamarai et al. [560] found CD34 expression to be significantly increased in leukemic cells from relapsed patients compared with newly diagnosed patients, and that in relapsed patients there was significant microenvironment mediated resistance to ATO which was demonstrated in vitro.

Hematopoietic stem cell transplantation for APL patients in second complete molecular remission is recommended by many investigators. Autologous stem cell transplantation is recommended rather than allogeneic transplantation [561].

APL and Pregnancy

The treatment of AML in pregnancy is discussed in general in Chap. 22. Although retinoids are well known to be teratogenic and their use during pregnancy has been advised against, reports of successful use of ATRA during the second and third trimesters in more than 40 patients have appeared [562–564] and no cases of teratogenic effects were observed. At least one case of successful pregnancy after ATO treatment has been reported [565]. Since ATRA rapidly controls the coagulopathy associated with APL, it may be an attractive induction agent for the APL patient in late pregnancy. Most women with APL treated with standard chemotherapy in late pregnancy have also survived and delivered normal children [566]. However, APL in the first trimester is likely to be associated with obstetric and fetal complications [567, 568].

APL in Children

APL in children generally has the same features as in adults, except that the incidence of leukocytosis and the microgranular variant is greater in children than in adults [569]. Children under the age of 5 years have a worse prognosis than older ones. APL in children is generally treated as in adults. The same agents used for adult APL are used in children, although many successful studies have employed lower ATRA doses (25 mg/m^2 daily), lower total anthracycline dose, and cytarbine in consolidation [427, 570]. ATRA toxicity is more frequent in children [IIII], particularly pseudotumor cerebri.

The Coagulopathy Associated with APL

A major feature distinguishing APL from all other subtypes of AML is the very frequent association with a severe life-threatening coagulopathy. The pathogenesis of

the coagulopathy is complex and includes disseminated intravascular coagulation (DIC), hyperfibrinolysis, proteolysis and exposure of tissue factor and annexin-II by leukemic blasts [571–573]. Historically, approximately 10–30% of patients with APL died of early fatal hemorrhage, often intracerebral hemorrhage [9, 409, 574–579]. Even in the modern era of therapy with *all-trans* retinoic acid (ATRA) plus idarubicin, hemorrhage remains the single most common cause of death before and after induction therapy [454]. This is particularly problematic since patients with APL historically [580–582] and now in the ATRA era are highly curable with ATRA plus anthracycline-based chemotherapy approaches or ATRA plus ATO regimens [506, 583]. Early death from coagulopathy has therefore emerged as the major obstacle to cure in APL [399, 584–590].

In every study to date, successful remission induction with ATRA has been accompanied by rapid resolution of clinical bleeding and generally, of biochemical evidence of the coagulopathy, although elevated plasma levels of sensitive markers of clotting activation may persist [446, 591, 592]. Studies of arsenic trioxide in patients with relapsed and refractory APL also show rapid correction of the clotting abnormality [182, 593]. The rapid resolution of coagulopathy in APL patients given ATRA and/or arsenic speaks to the importance of prompt treatment in order to prevent early death from coagulopathy [573, 584, 589].

Coagulopathy and Early Death in APL

Approximately 70–80% of patients with either previously untreated APL or relapsed disease have either laboratory or clinical evidence of a potentially life-threatening bleeding diathesis [9, 182, 354, 409, 415, 418, 574–578, 594]. Even in the modern era, the risk of hemorrhagic death during induction remains at about 5–9% in the controlled environment of clinical trials, and may be as high as 16–31% in population studies [399, 402, 454, 534, 542, 573, 585–587, 590, 595, 596] (Table 21.3). Clinical manifestations of coagulopathy in APL can include mucocutaneous bleeding in the form of ecchymoses, petechiae, epistaxis, and gastrointestinal hemorrhage, as well as increased bleeding at sites of minor trauma or catheter insertion [573]. Intracranial and pulmonary hemorrhages are the most feared manifestations of coagulopathy in APL, as these two sites of bleeding account for the majority of hemorrhagic deaths in most series [402, 454, 573, 584, 585, 588, 590, 597]. In the Spanish PETHEMA study, intracranial and pulmonary hemorrhage accounted for 65% and 32% of hemorrhagic deaths [454]. Similarly in the Japanese JALSG APL97 study, intracranial and pulmonary hemorrhages accounted for 66% and 22% of early deaths [597].

In addition to bleeding, some studies suggest that patients with APL are also more prone to thrombosis than patients with other leukemias [584]. Various thrombotic events have been documented in APL patients, including myocardial infarction, deep vein thromboses, pulmonary emboli, and CNS thromboses [584, 599–604]. The incidence of thrombo-embolic deaths in APL patients ranges from 5.1% to 9.6% in various studies [18, 42–44]. Thromboembolism in APL patients may be associated with procoagulant effects of ATRA and worsened by differentiation syndrome [584, 605, 606].

Pathophysiology of the Coagulopathy in APL

The etiology of coagulopathy in APL is complex, with multiple driving mechanisms including tissue factor-induced DIC and primary hyperfibrinolysis [573]. The characteristic pattern of laboratory abnormalities includes thrombocytopenia; prolongations of the prothrombin time (PT), partial thromboplastin time (PTT), and thrombin times; increased levels of fibrin degradation products; and hypofibrinogenemia [594, 607–610]. These findings are consistent with either DIC and hyperfibrinolysis or both. Notably, patients with APL can have potentially fatal bleeding even in the absence of abnormal PT and PTT. In addition, although APL patients classically experience profound hypofibrinogenemia, some individuals with APL do not have significantly decreased fibrinogen [573, 594]. Furthermore, levels of several anticoagulant proteins such as antithrombin III and protein C, often low in the setting of DIC, are usually not decreased in patients with APL [611]. Platelet survival in these patients is normal, reflecting a more complex process than DIC alone [612, 613].

Multiple procoagulant mediators have been described in patients with APL (Fig. 21.7) [622–624]. Tissue factor (TF) is the major procoagulant that initiates the extrinsic pathway of

Table 21.3 Early death rate and bleeding in APL in prospective cooperative group studies

Trial	*N*	Induction	CR%	ED%	ED from bleeding%	DFS%
PETHEMA [580, 598]	732	ATRA + Ida	91	9	56	84
JALSG [542]	283	ATRA/Ida/ara-C	94	5	69	69
GAMLCG [534]	142	ATRA/TAD/HAM	92	8	64	82
GIMEMA [596]	420	ATRA + Ida	94	6	32	87

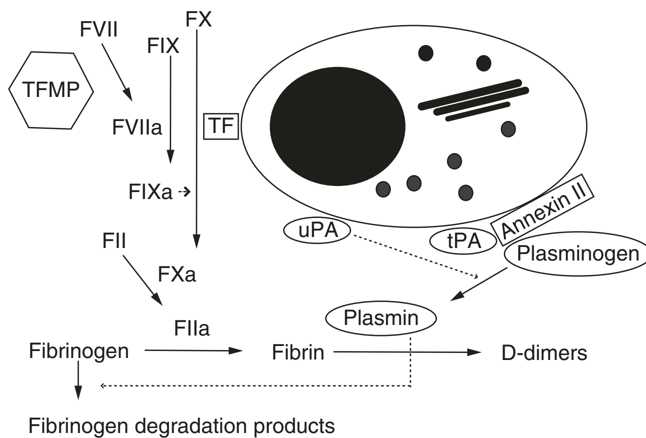


Fig. 21.7 Simplified schema of the coagulopathy associated with APL. Tissue factor present at the surface of leukemic blasts and microparticles binds to factor (F)VII, leading to activation of the latter. The TF-FVIIa complex activates FIX and FX. This leads to thrombin generation, which itself catalyzes fibrin formation. Parallel to this coagulation activation and factor consumption process, Annexin II present at the surface of malignant leukocytes binds tPA and plasminogen, resulting in the formation of plasmin, which goes on to cleave fibrin and fibrinogen. *APL* acute promyelocytic leukemia, *FII* coagulation factor II (prothrombin), *FIX* coagulation factor IX, *FVII* coagulation factor VII, *FX* coagulation factor X, *TF* tissue factor, *TFMP* tissue factor microparticle; *tPA* tissue plasminogen activator; *uPA* urokinase-type plasminogen activator. Reproduced with permission from Mantha S et al., Current Opinion in Hematology 2016 [573]

blood coagulation in vivo and is the membrane protein receptor for factor VII [614]. The resulting TF-factor VIIa complex activates factors IX and X, which leads to thrombin generation and fibrin formation. The TF gene is expressed in cells from patients with APL, and the TF promoter is activated by the PML/RARα fusion protein [615–618]. Recent work has also described tissue factor microparticles (TFMP) as potentially important for the pathogenesis of coagulopathy in APL [573, 619–621]. TFMP are cellular membrane fragments displaying TF derived from tumor cells, platelets, endothelial cells and monocytes. As in solid tumors, TFMP in APL manifest procoagulant activity as evidenced by measurements of thrombin generation [573, 619–621]. Increased expression of the cysteine proteinase cancer procoagulant (CP) protein by APL blasts has also been proposed as an additional mechanism of coagulopathy in APL [574–576]. Cytokines such as interleukin-1 (IL-1), tumor necrosis factor (TNF), and vascular permeability factor (VPF) are indirect procoagulants in APL by initiating coagulation through the induction of TF in endothelial cells and monocytes [625–627]. Interleukin-1 secreted by leukemic cells may induce DIC [628, 629]. Cytokines can generate plasminogen activator inhibitors that inhibit vessel wall fibrinolytic activity promoting coagulation [630, 631]. Interferon-γ and VPF-like mediators can induce endothelial cell procoagulant activity [632, 633].

Excessive fibrinolysis is also an important factor in the coagulopathy in APL [634, 635]. Plasminogen and alpha-2 antiplasmin levels are reduced in patients with APL [636, 637]. Furthermore, leukemic promyelocytes release plasminogen activators that cleave plasminogen and initiate fibrinolysis. Circulating tissue-type plasminogen activator can be found in the plasma of some patients with APL [638]. Decreased levels of circulating plasminogen-activator inhibitor type 1 (PAI-1) have been reported in some patients [639, 640]. APL cells contain elastases that inactivate alpha-2-plasmin inhibitor [641]. Annexin-VIII is one of a group of naturally occurring proteins that bind phospholipids and have both anticoagulant and phospholipase-A2 inhibitory properties [642]. The Annexin-VIII gene is expressed to a greater degree in cells from patients with APL compared to cells from patients with other subtypes of AML [643]. Annexin-VIII is highly expressed in the APL cell line NB4 and is significantly reduced after exposure to ATRA. Annexin-II is a cell surface receptor for plasminogen and its activator, tissue plasminogen activator (t-PA), which functions as a t-PA cofactor [644, 645]. Annexin-II is expressed in high levels on leukemic promyelocytes compared to leukemic cells from patients with other subtypes of AML [644, 645]. In APL, Annexin-II may play an important role in the formation of plasmin, serving as a driver for intense fibrinolytic activity [573, 644, 646, 647]. Studies suggest that plasmin- and elastase-induced degradation of von Willebrand factor also contributes to the hemostatic defect in APL [648].

Influence of ATRA on Coagulation Parameters

The effects of ATRA on APL coagulopathy are complex and incompletely understood. A number of studies have examined the specific changes in coagulation parameters before and after ATRA. Dombret and colleagues studied a small number of patients with APL treated with ATRA and reported that both DIC and proteolysis improved within 14 days [581]. Although proteolysis appeared to completely resolve, low-grade procoagulant activity persisted, even after patients achieved complete response, particularly in patients who developed hyperleukocytosis during treatment. Markers of thrombin generation such as thrombin–antithrombin complex (TAT), prothrombin fragment 1 + 2 (F1 + 2), and D-dimer did not completely normalize. These findings reflect a dissociation between the resolution of proteolysis (fibrinogenolysis) and DIC and may explain some reports of thromboembolic events during treatment with ATRA, particularly when ATRA is combined with antifibrinolytic therapy as prophylaxis against bleeding [584, 602, 605, 606, 649, 650].

Both TF-like and factor VII-independent (CP-like) procoagulant activity of APL blast cells is decreased after ATRA exposure [651, 652]. ATRA both upregulates thrombomodulin and downregulates TF expression in APL cells [654, 698]. However, several studies have demonstrated that retinoic acid also stimulates tissue-type plasminogen activator in human endothelial cells, which can initiate the fibrinolytic cascade and counterbalance the effects of a decrease in other procoagulant mediators [655, 656]. Levels of markers of coagulation activation including D-dimer, F1 + 2, TAT, and fibrinopeptide A have been shown to decline following exposure to chemotherapy or ATRA. Notably, plasma levels of D-dimer, F1 + 2, TAT, and fibrinopeptide-A decreased more rapidly among patients treated with ATRA compared to chemotherapy. D-dimer, TAT, and fibrinopeptide A levels also remain significantly elevated well above the upper limit of normal among the chemotherapy-treated patients [592]. Later in the course following ATRA and chemotherapy treatment, re-elevation of several molecular markers of coagulation may occur in some patients, potentially attributable to late effects of cytotoxic chemotherapy and infection [582]. Overall, most studies support the unifying hypothesis that as ATRA induces terminal differentiation of leukemic promyelocytes, markers of both procoagulant activity and fibrinolytic activity decrease with some evidence of persistent mild DIC.

Effect of the Improvement in the Bleeding Diathesis on Outcome of APL Patients Treated with ATRA or Arsenic

Nonrandomized and randomized prospective trials have examined the outcome of patients treated with ATRA alone or with ATRA and chemotherapy [267, 402, 430, 539, 657]. Before the ATRA era, the risk of early hemorrhagic death for APL patients ranged from 10 to 30% [9, 409, 574–579]. However, since the introduction of ATRA in 1988, the risk of early hemorrhagic death in prospective APL studies has decreased significantly to 5–10% [266, 402, 408, 430, 445, 539, 573, 658–661].

Arsenic trioxide also has emerged as an important agent in the treatment of APL, with recent studies demonstrating that ATRA plus arsenic trioxide is as at least noninferior and possibly superior to ATRA plus chemotherapy in the treatment of patients with low-to-intermediate-risk APL [506]. Unlike ATRA, arsenic trioxide binds the APL oncoprotein and leads to its degradation, resulting in decreased transcription of downstream target genes, suggesting that it could ameliorate the coagulopathy of APL early in its pathogenesis [63, 190, 573, 662]. Arsenic trioxide induces rapid loss of membrane procoagulant activity and TF mRNA [663]. Preliminary studies have shown that arsenic trioxide also has a beneficial effect on the coagulopathy in APL [605, 664]. Given the excellent outcomes in modern APL prospective clinical trials, it is difficult to assess the impact of arsenic in decreasing bleeding in contemporary studies. For example, there were no cases of early death in the ATRA + arsenic trioxide arm of the recent study comparing ATRA + arsenic and ATRA + chemotherapy in low-to-intermediate risk APL. However, there was only one death from hemorrhagic shock in the ATRA + chemotherapy group. Overall, patients with low-to-intermediate risk APL treated either with ATRA + arsenic or ATRA + chemotherapy had very good outcomes [506]. It is possible that there may be more room for improvement in reducing risk of early death from coagulopathy in high-risk APL. Since the mechanism of induction of remission of arsenic trioxide appears to be different from that of ATRA, new opportunities are present to explore the pathogenesis of the coagulopathy and the pathophysiologic basis for its improvement.

The Next Frontier: Management of Coagulopathy and Prevention of Early Death in APL

Despite the dramatic improvements in APL outcomes and reduced rates of hemorrhage in clinical trial settings, early death from hemorrhage remains a significant problem in population and community studies [399, 454, 573, 585, 587, 588, 590, 595] (Table 21.4). As early death in APL is driven significantly by coagulopathy, management of the coagulopathy of APL is therefore critical to ensuring high rates of cure [573, 584, 589] (Table 21.5). The most important initial step in managing the coagulopathy of APL involves suspecting the diagnosis of APL and prompt administration of ATRA. Given the rapid impact of ATRA in altering coagulation parameters

Table 21.4 Early death rate in APL in population studies

Study	*N*	ED%
Jeddi [595]	41	16
Lehmann [585]	99	31
McClellan [588]	70	26
Park [590]	1400	18

Table 21.5 Management of coagulopathy and prevention of early hemorrhagic death in APL

• Start ATRA at first suspicion of APL (based on clinical history and review of peripheral smear), BEFORE BONE MARROW AND BEFORE DIAGNOSIS CONFIRMED (in ER)
• Frequent platelet transfusion to >50,000/μL
• Cryoprecipitate to maintain fibrinogen >150 mg/dL
• No heparin, although not studied in ATRA era
• No antifibrinolytics

as noted above, ATRA should be given whenever APL is first suspected following review of the peripheral smear—without waiting for genetic confirmation of APL diagnosis and without waiting for bone marrow biopsy [257].

In addition to rapid administration of ATRA, vigilant monitoring of coagulation parameters and frequent administration of blood products are needed to prevent early hemorrhagic death in APL. Blood products serve as the cornerstone or prohemostatic treatments in APL, including platelet transfusions, fresh frozen plasma, as well as cryoprecipitate for repletion of fibrinogen [257, 573]. Guidelines support maintaining the platelet count above 30,000–50,000/μL and maintaining fibrinogen above 100–150 mg/dL (Table 21.5) [257]. Replacement therapy should be continued until all clinical and laboratory signs of coagulopathy have resolved. Although all patients presenting with APL are potentially at risk of hemorrhage, specific factors predicting high risk of fatal hemorrhage have been identified. In particular, patients with active bleeding, increased WBC or peripheral blast counts, abnormal renal function, poor performance status, hypofibrinogenemia (<100 mg/dL), or increased levels of fibrin degradation products combined with prolonged prothrombin or activated partial thromboplastin time are at increased risk of developing fatal hemorrhage [257, 267, 402, 454, 597, 660].

Beyond ATRA and blood products, there are no robust data to support the use of other prohemostatic modalities in APL. Intravenous heparin, antifibrinolytics, recombinant factor VIIa, and thrombomodulin have all been tested in APL patients, though none have been tested in randomized trials in the ATRA era [573]. Although heparin was advocated prior to the advent of ATRA, there are concerns as to the safety of heparin due to the possibility of worsening bleeding. Alternate concerns have been raised regarding the use of antifibrinolytics, due to the potential of thrombotic complications and the known predisposition of APL patients to thrombosis [584, 599–604]. Recombinant factor VIIa has been described in case reports of life-threatening hemorrhage in APL patients [665, 666]. However, the data for recombinant favor VIIa in this setting is highly limited, and recombinant factor VIIa presents similar safety concerns as antifibrinolytics in serving as a potential trigger for thrombosis. Recent retrospective postmarketing data from Japan suggests that the natural anticoagulant thrombomodulin may be useful in treating the coagulopathy of APL [667]. However, although the 3.5% rate of hemorrhagic death in APL patients who receive thrombomodulin is encouraging and lower than rates of hemorrhagic death in community studies, there are no randomized prospective trials of thrombomodulin that demonstrate clear efficacy in the treatment of coagulopathy in APL. Therefore, we would not recommend the use of thrombomodulin for treatment of APL coagulopathy outside of the context of clinical trials.

Management of coagulopathy in APL involves prompt suspicion of APL diagnosis, rapid administration of ATRA, and vigilant use of blood products. Given the disparity in rates of early hemorrhagic death between APL patients in population studies and in prospective academic clinical trials, education of community physicians in recognition and management of the coagulopathy of APL will be critical to improving overall rates of cure.

Future Treatment Research

Although tremendous progress has been made in the treatment of APL in the last two decades, problems remain. The coagulopathy characteristic of APL is not sufficiently addressed by the advent of ATRA or ATO. Mechanisms to interrupt the coagulopathy more rapidly need to be developed. Therapy, especially for high-risk patients needs to be improved. It is still unclear whether ATRA plus ATO or ATRA plus anthracycline is the best induction treatment for high-risk patients and experts have called for a randomized trial to address that issue [668]. A number of potential improvements in treatment are currently under investigation. Zoldronic acid was shown by Liu et al [669] to synergize with ATO in the inhibition of NB4 cells in vitro and should be explored clinically. Homoharringtonine [670] plus ATRA induction therapy followed by consolidation therapy and a 2-year maintenance program produced a 100% 9-year overall survival among patients with APL who were not overweight in one study, while obese patients had only a 73% 9-year survival rate. Other studies have shown that APL patients were more likely to be obese than other patients with AML. The effect of obesity on APL needs further study, and homoharringtonine as therapy for APL deserves further study as well. pVAX14DNA-mediated immunotherapy together with ATRA and ATO increased the survival of APL in mice [671]. This is an interesting observation that should also be explored further. AZD11512, a specific inhibitor of Aurora B which is overexpressed in APL, showed significant activity against NB4 cells and could lead to an entirely new approach to treatment of APL [672].

ATO degrades PML-RARα oncoprotein via the proteasome pathyway. Ganesan et al. [673] demonstrated in the laboratory that ATO and bortezomib (a proteasome inhibitor) are synergistic against arsenic sensitive and resistant cell lines. They have begun a test of this finding in the clinic. A traditional Chinese agent, tanshinone, was shown to induce differentiation in APL cells and subsequently shown to significantly prolong survival of APL-bearing mice [674]. Clinical trials should be entertained. Fucoidan, a sulfated polysaccharide from brown algae, apparently enhanced the activity of ATRA or ATO in a mouse model of APL [675] and deserves further study.

These early studies are interesting and may lead to newer approaches to the treatment of APL in the future.

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Therapy-Related Acute Myelogenous Leukemia

22

Hyung Chan Suh and H. Phillip Koeffler

Abbreviations

AD	Autoimmune disease
ADC	Antibody-drug conjugate
ALL	Acute lymphoblastic leukemia
AlloHSCT	Allogeneic hematopoietic stem cell transplant
AML	Acute myelogenous leukemia
ATRA	All-trans retinoic acid
AutoHSCT	Autologous hematopoietic stem cell transplant
CLL	Chronic lymphocytic leukemia
CR	Complete remission
ECOG	Eastern Cooperative Oncology Group
EGR-1	Early growth response-1
FLT3	fms-related tyrosine kinase 3
FPSG	French Polycythemia Study Group
G-CSF	Granulocyte colony-stimulating factor
GM-CSF	Granulocyte-macrophage colony-stimulating factor
HDAC	Histone deacetylation
HLA	Human leukocyte antigen
HSC	Hematopoietic stem and progenitor cells
IL	Interleukins
LDH	Lactate dehydrogenase
M-CSF	Macrophage colony-stimulating factor
MDR	Multiple drug resistance
MF	Myelofibrosis
MLL	Mixed-lineage leukemia
MPN	Myleoproliferative neoplasia
MRI	Magnetic resonance imaging
MUGA	Multigated acquisition scan
NHL	Non-Hodgkin lymphoma
OS	Overall survival
PBSC	Peripheral blood stem cells
PDGF	Platelet-derived growth factor
PET	Positron emission tomography
PLK	Polo-like kinase
PML	Promyelocytic leukemia protein
PVSG	Polycythemia Vera Study Group
RAEB	Refractory anemia with excess blasts
RAEB-t	Refractory anemia with excess blasts in transformation
RARA	Retinoic acid receptor alpha
RIC	Reduced-intensity chemotherapy
SCN	Severe congenital neutropenia
T-AML	Therapy-related acute myelogenous leukemia
T-APL	Therapy-related acute promyelocytic leukemia
T-MDS	Therapy-related myelodysplastic syndrome
T-MN	Therapy-related myeloid neoplasm
TNF	Tumor necrosis factor
TP53	Tumor protein p53
TRM	Treatment-related mortality
WBC	White blood cell

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Introduction

Individuals exposed to cytotoxic agents are at higher risk of developing myeloid disorders such as therapy-related myelodysplastic syndrome (t-MDS), therapy-related acute myeloid leukemia (t-AML), and therapy-related MDS/myeloproliferative neoplasms. However, all of these diseases are within the spectrum of a single disease entity, therapy-related myeloid neoplasms (t-MN), as categorized by the WHO classification system in 2008 [1]. WHO morphologic classification system defines t-MN as MDS and myeloid leukemia, which arise following the administration of chemotherapy and/or radiation for a prior malignancy. Patients who developed myeloid disorders by environmental toxins affecting hematopoiesis are not included in this disease category. Therapy-related MDS

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and AML comprise the vast majority of t-MN cases. The 2008 WHO classification did not consider t-MDS and t-AML sufficiently distinctively different. However, unlike secondary AML denoting AML did not develop spontaneously or *de novo*, t-AML has clear history of prior chemotherapy or radiation therapy.

T-MN has become increasingly common. The fast rising incidence can be attributed to a variety of factors including longer survival of patients after treatment of their primary malignancy, intensified chemotherapy, radiation therapy, and broaden awareness of this disease category. This is a heterogeneous and poorly defined group of patients who have a shorter median survival than patients with *de novo* AML, MDS, or MDS/myeloproliferative neoplasia (MPN). Retrospective studies have shown that their inferior outcomes are associated with poor-risk cytogenetics, present in 50–70% of t-MDS/AML compared with 15–25% in *de novo* disease. Other studies have identified additional risk factors, including comorbidities from primary malignancy and therapy of the disease. Because of the poor outcome, t-AML is among the most feared long-term complication of cancer therapy these days.

Epidemiology

Therapy-related myeloid neoplasms (t-MN) account for approximately 10–20% of all cases of AML, MDS, and MDS/MPN. US Surveillance, Epidemiology, and End Results data of approximately 426,000 adults treated for an initial primary malignancy between 1975 and 2008 showed a 4.7-fold increased risk of AML compared with the incidence of AML expected in the general population. With the increasingly successful management of malignancies overall and improved cancer survivorship, the overall incidence of t-MN is expected to increase. The estimated incidence after therapy for any single prior diagnosis varies from less than 1 to 20% depending on the agents administered, therapy intensity, and survival, since the overall median latency time varies 1–5 years.

Patients with t-AML are seen among survivors of both solid tumors and hematologic malignancies. Smith et al. studied 306 patients who developed therapy-related myelodysplasia and myeloid leukemia with cytogenetic analyses [2]. In the study population, 25% of the patients had Hodgkin disease, 23% had non-Hodgkin lymphoma, and 38% had a solid tumor as the primary malignancy. Breast cancer was the most common among the 38% patients. Interestingly, 6% of patients had undergone cytotoxic chemotherapy for the management of immune disorders. Kayser et al. also showed similar patient characteristics in their study with 200 patients having t-AML. The group found that 71% of t-MN patients had a prior solid tumor and 27.5% patients had a prior hematologic malignancy. Breast cancer and non-Hodgkin lymphoma were the largest subsets in these two groups [3].

T-AML patients can present at any age. The risk associated with alkylating agents and radiation appears to increase with age, while the risk associated with topoisomerase II inhibitors appears to be constant across all ages [1]. Among those treated for breast cancer, younger age at the time of exposure, higher dose intensity of cytotoxic treatments, concomitant treatment with radiation, and adjuvant use of hematopoietic growth factors with cytotoxic therapy for accelerated white blood cell recovery are factors associated with an increased risk of t-AML [4, 5]. However, some t-AML/MDS individuals may have a DNA repair apparatus that is not as robust as normal, which also might predispose them to develop t-AML.

Etiology

T-AML appears to be a direct consequence of mutational events by therapy-induced DNA double-strand breaks, with a subsequent genomic instability [6]. Frequency of the mutations may vary between individuals as a result of genetic susceptibility. This susceptibility is usually not measurable or very subtle; a few exceptions include Fanconi anemia, and mismatch repair abnormalities.

The effects of some cytotoxic agents in the development of abnormal cytogenetics are well documented (Table 22.1). The latency period between first exposure to an agent (cytotoxic chemotherapy, radiation) and development of t-AML ranges from 1 to 5 years and varies by etiologic agent. T-AML after exposure to alkylating agents or radiation therapy typically presents after a latency period of approximately 4 years [7–10]. Most of these patients initially present with MDS. The chromosomal abnormalities seen in this category of t-AML often involve complex abnormalities such as deletion of the long arm or the entire chromosome 5 and/or 7. Topoisomerase II inhibitors are another etiologic agent of t-AML. It causes t-AML with a relatively shorter latency of

Table 22.1 Risk factors for therapy-related leukemia

Alkylating agent therapy: May cause MDS (preleukemic phase), could take 4–10 years to develop AML 5q or 7q deletion, bad prognosis
DNA-topoisomerase II inhibitor therapy (epipodophyllotoxins and anthracyclines): May develop t-AML without preleukemic phase; short median latency (33 months) Frequent translocation of 11q23 (MLL) or 21q22 Morphologic phenotype often M4/M5 (by former FAB classification)
Ionizing radiation therapy: similar to alkylating-related AML
G-CSF in severe congenital neutropenia

1–3 years, and the patients present with overt leukemia rather than MDS or MDS/MPN [11–13]. The cytogenetic alterations in this category of t-AML occur frequently with translocations including the MLL gene located at 11q23 or AML1 (RUNX1) gene at 21q22 [e.g., t(9;11), t(8;21), or t(3;21)]. However, no reliable way exists to determine the duration of the "at-risk" period for developing t-AML. The latency periods with other agents are not as clear as these two drugs. Exposure to multiple agents also makes it difficult to determine the risk, etiology, and latency period.

Alkylating agents are frequently used chemotherapeutic agents; more than 85% of patients who developed chemotherapy-related leukemia had received an alkylator [14]. Melphalan, chlorambucil, or cyclophosphamide is the offending agent in nearly 65% of patients. Therefore, different alkylating agents may be associated with varying risks of leukemogenesis. For example, one study compared the rates of mutagen-related leukemia in ovarian cancer patients treated with either melphalan or cyclophosphamide, and found that melphalan may be a more potent leukemogen than cyclophosphamide. Thus, the mutagenic potential may differ between the antineoplastic agents [15].

Alkylating agents interact with DNA in a variety of ways: monoadduct formation, inter- and intra-strand cross-links, as well as alkylation of free DNA bases. This can lead to cell death, but also can cause termination of DNA replication and chromosome loss, leading to mutagenesis and resulting in development of leukemia. Alkylation events can also change the stereometric configuration of DNA bases, causing them to mispair resulting in single-base mutations. Many of the alkylating agents have been clearly implicated in leukemogenesis.

Topoisomerase II helps mediate the relaxation of the DNA supercoil by making double-strand breaks. The breaks are repaired when homologous chromosome fragments realign. Topoisomerase II inhibitors, such as epipodophyllotoxins (etoposide and teniposide), doxorubicin, 4-epodoxorubicin, mitoxantrone, razosane, and biomolane, induce incorrect DNA repair by crossover recombination with nonhomologous end joining between the two DNA strands, which may result in the development of a balanced chromosomal translocation (Fig. 22.1). Balanced chromosomal aberrations involving the MLL, RUNX1, RARA, or NUP98 genes characterize unique genetic pathways of t-AML. The rearrangements between these genes and other partners provide gain-of-function fusion proteins. These topoisomerase II inhibitors are important components of chemotherapy regimens for many tumors, such as testicular cancer, ALL, NHL, lung cancer, and many others. Razoxane and bimolane, used in the treatment of psoriasis, have also been demonstrated to be leukemogenic. Those patients in whom t-AML develops after therapy with DNA-topoisomerase II inhibitors often have acute leukemia with no t-MDS phase [16, 17], and a short latency period, in contrast to alkylating agent-induced AML.

In the Polycythemia Vera Study Group (PVSG), 431 polycythemia vera patients were randomized to one of the three treatment groups: phlebotomy alone, P32 and phlebotomy, or chlorambucil and phlebotomy [18]. Higher number of AML cases occurred in both the P32 (9.6%) and chlorambucil (13%) treatment groups compared to phlebotomy-only group (1.5%), indicating a role of radiation and a cytotoxic agent in the development of AML in these patient groups [19]. French Polycythemia Study Group (FPSG) reported a leukemia incidence in polycythemia vera patients of 5–15% after 10 years of observation [20]. In a randomized trial in patients >65 years of age, the FPSG reported 12% AML at 10 years in patients receiving P32 alone. Hydroxyurea

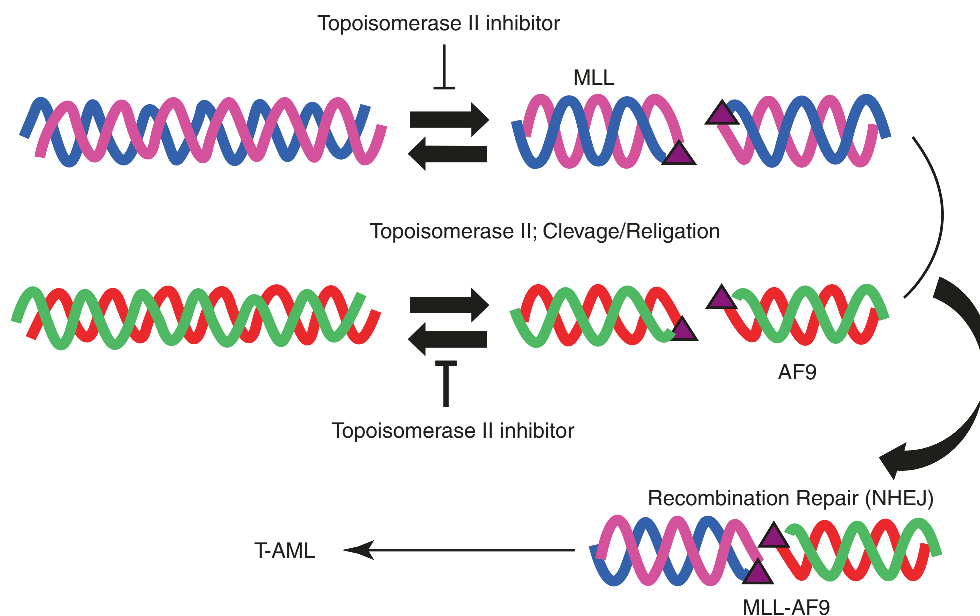

Fig. 22.1 Formation of topoisomerase II-DNA complex is necessary to perform critical cellular functions. If the amount of complexes is elevated as a result of topoisomerase II inhibitors, DNA repair/recombination process is activated, which subsequently generates chromosomal translocations or other DNA aberrations. If the fusion protein produced by chromosomal translocation results in the cells having a growth advantage, these cells may evolve and progress into t-AML

maintenance combined with initial P32 therapy also increased the risk of AML (21% at 10 years). In another case control study of MPN patients (68% of patients had polycythemia vera), the risk of AML/MDS development was significantly associated with high exposures of P32 and alkylators [21]. Taken together, a strong association exists of cytotoxic agents or radiation increasing the risk of development of AML in MPN patients.

Ionizing radiation clearly increases the risk of developing AML in humans and experimental animals. The incidence of leukemia after 400 cGy or less of radiation exposure from the Hiroshima nuclear explosion was approximately two cases of leukemia/10^6 persons/year/cGy [22]. Nearly the same incidence of leukemia was reported in patients who received 300–1500 cGy of spinal irradiation for ankylosing spondylitis. Likewise, increased rates of AML occurred in radiologists who practiced during the early years of clinical radiology before modern safety standards [23].

Animal studies confirmed the epidemiological observations in humans by showing that low-dose chronic irradiation induces leukemia in experimental animals. Half of dogs that received a daily low dose (5–10 cGy) of cobalt γ-irradiation developed AML after about 1000 days [24]. Single whole-body irradiation initiates leukemia in rodents. Myelogenous leukemia developed in 20% of mice after a single brief whole-body irradiation of 200 cGy [25]. The dose–response relationship was curvilinear; pulse irradiation of at least 300 cGy induced significantly fewer cases of leukemia than the 200 cGy dose; these doses produced marrow cell death, probably decreasing the number of cells that would otherwise have the potential to undergo malignant transformation.

Exposure to ionizing radiation can cause a DNA damage by a mechanism similar to alkylating agents. Radiation photon energy can directly lead to DNA strand breakage. Radiation is frequently used in conjunction with chemotherapy for cancer therapy, and only a few studies have specifically looked at the characteristics of myeloid neoplasms occurring after radiation alone. Recently, Nardi et al. showed that t-MDS occurring in the modern radiation therapy era, if alone, more nearly resembled *de novo* MDS/AML in cytogenetic characteristics and clinical behavior, and affected patients had better outcomes than patients with t-MDS secondary to chemotherapy [26].

Even though radiation therapy is leukemogenic [7, 27–29], studies in Hodgkin's disease suggest that the incidence of secondary leukemia in patients receiving radiation therapy alone was low compared with those receiving chemotherapy alone [30, 31]. In one study, a total of 957 patients exclusively received radiation therapy, and none developed leukemia. By contrast, 542 patients received only chemotherapy, and 12 developed leukemia. A similar finding was reported in ovarian cancer patients who received either chemotherapy or radiation therapy [32]. In most studies, the risk of AML in patients with either Hodgkin's disease or ovarian carcinoma treated exclusively with chemotherapy is not different from those treated with both chemotherapy and radiation therapy [7, 27–29, 32].

In a study of chromosomal abnormalities, only 37 of 344 patients with secondary leukemia had been treated with radiation therapy alone; the incidence of a normal karyotype was higher in patients who received only radiation than in patients who received chemotherapy either with or without radiation therapy (24.3 vs. 11.7%). Normal karyotype is associated with better response to antileukemic therapy, but with little improvement in overall survival (OS) [33]. Another study of 63 patients with either t-MDS or t-AML found that 11 of 63 had received only radiation, in most cases to ports including the pelvis or spinal bone marrow [34]. In this study, only two patients had a normal karyotype. The low risk of leukemia after currently used high-voltage irradiation may be analogous to the earlier mentioned murine model where high-dose irradiation has a lethal effect on marrow cells in contrast to lower dose exposure, which may be more likely to produce nonlethal marrow cell injury and mutations.

Although several studies examined secondary malignancies in patients with specific primary tumor types, few data have been published examining the long-term effect of pelvic radiation. Wright et al. analyzed patients with invasive tumors of the vulva, cervix, uterus, anus, and rectosigmoid treated with radiotherapy from 1973 to 2005 [35]. In a Cox proportional hazards model adjusting for other risk factors, posttreatment leukemia was increased by 72% (hazard ratio [HR], 1.72; 95% CI, 1.37–2.15) in the patients who received pelvic radiotherapy. The risk of secondary leukemia peaked at 5–10 years after primary treatment (HR, 1.85; 95% CI, 1.40–2.44) and remained elevated even 10–15 years after initial treatment (HR, 1.50; 95% CI, 1.03–2.18) [35].

Radioiodine (I-131) induces chromosomal aberrations, and theoretically can lead to leukemogenesis. However, the occurrence of t-AML after radioiodine treatment for thyrotoxicosis and thyroid cancer is infrequent. In a comprehensive meta-analysis of the currently available literature covering 16,502 patients with thyroid cancers, the relative risk of development of leukemia increased 2.5-fold in patients treated with radioiodine [36]. The latency period of t-AML associated with radiation was 5–7 years, similar to t-AML associated with alkylating agents [37].

The use of granulocyte colony-stimulating factor (G-CSF) in chemotherapy may be a risk factor for development of t-AML as shown in a meta-analysis examining data from 25 trials [38]. At a mean follow-up of 60 months, 43 t-MN cases were reported in G-CSF-treated patients, while 22 t-MN occurred in control group. G-CSF may accelerate damaged myeloid progenitors into cell cycling before repair of genetic injuries from cytotoxic therapy.

Cases of leukemic transformation in patients with severe congenital neutropenia (SCN) were prospectively studied [39]. A comprehensive analysis of the incidence of AML transformation showed that the annual risk of MDS/AML was 0.81% during the first 5 years, and 2.3% after 10 years among 374 SCN patients with G-CSF treatment. After 15 years on G-CSF, the cumulative incidence for MDS/AML was 22% in SCN, whereas none of the cyclic neutropenia patients who also received G-CSF developed MDS or AML [40]. Patients with SCN develop mutations of their G-CSF receptor, which affects the ability of the myeloid cells to differentiate.

T-MN occurring in patients with autoimmune diseases (AD) has been increasingly recognized. A large population-based study found that AD patients had significantly increased risk for AML and MDS [41], and this finding was subsequently confirmed by another study [42]. Immunosuppressive therapy may be another contributing factor for development of t-MN. Patients who received immune-suppressive agents including corticosteroids, antitumor necrosis factor (TNF) agents, sulfasalazine, and cytotoxic chemotherapeutics such as methotrexate, azathioprine, and cyclophosphamide had increased risk for hematological malignancies [43]. The development of t-APL in patients with multiple sclerosis has been reported [44, 45]. But patients receiving an antimetabolite as a single agent (e.g., fludarabine, azathioprine, and 6-thioguanine) for their autoimmune disease rarely develop t-AML [46–48]. Development of t-AML in AD patients who received immunosuppressive therapy other than cytotoxic agents could represent the importance of the immune-surveillance system in guarding against malignancies. Also, the underlying primary genetic defects in these individuals might increase susceptibility to AML.

Karyotypic Abnormalities in t-MDS/AML

Clonal chromosomal abnormalities can be detected in the blast cells of 80–95% of t-MDS/t-AML patients by routinely available techniques [7, 34, 49–53]. A hypodiploid modal number of chromosomes occur most frequently in t-MDS/t-AML patients. Hyperdiploidy, mainly trisomy 8, is rare and is often observed as an inconsistent aberration present in only a subclone of cells [52]. Chromosomes 5q and 7q probably contain critical myeloid tumor-suppressor genes in *de novo* and t-AML. The breakpoints for the deletions are variable, but a common chromosome region, the so-called critical region, is almost always deleted.

For chromosome 5, Le Beau et al. have narrowed down the critical region to 5q31.1, which includes the early growth response gene *(EGR-1)* [54]. Other genes located on the long arm of chromosome 5 include many growth factor genes, namely *granulocyte-macrophage colony-stimulating factor* (*GM-CSF*), and *interleukins-3, −4*, and *−5 (IL-3, −4, −5*) [34, 55–58], and the growth factor receptor genes known to be present on the long arm of chromosome 5, namely *macrophage colony-stimulating factor* (M-CSF or *FMS) receptor, platelet-derived growth factor (PDGF) receptor*, *glucocorticoid receptor, alpha1-adrenergic receptor, beta2-adrenergic receptor*, and *D1-dopamine receptor* [59, 60].

The breakpoints for the deletions of 7q are variable, but a common chromosome region, the so-called critical region, is located at band 7q22 proximally with the distal breakpoint varying from q31 to q36. Potentially important genes have been mapped to 7q, including genes for EZH2, erythropoietin, p glycoprotein 1/multiple drug resistance 1 (MDR-1), and MDR-3 [61]. Abnormalities of chromosome 7q are common in myeloid malignancies. Especially, homozygous EZH2 mutations were commonly found in MDS/MPN patients [61]. However, none has yet been shown to be involved in the development of t-AML [54, 59–62].

Although most of the chromosomal abnormalities reported in t-MDS/t-AML are either complete or partial deletion of chromosome 7 or 7q [del(−7/7q)], and/or 5, or 5q del(−5/5q)], in recent years recurring unbalanced translocations that also result in loss of the long arm of 7 and/or 5 have been reported with increasing frequency. These include t(1;7)(p11;q11), t(5;7)(q11.2;p11.2), and t(7;17)(p11;p11) for chromosome 7, and t(5;7)(q11.2;p11.2) and t(5;17)(p11;p11) for chromosome 5 [63]. While the loss of function of a single gene in each of these relatively large regions is possibly responsible for the development of t-MDS/t-AML, hemizygous loss of the function of several genes in each of these regions could also contribute to the disease phenotype. Another, not mutually exclusive hypothesis is that an unknown initiating abnormality causes genomic instability leading to the deletion and rearrangement of particularly susceptible chromosome regions, such as those on chromosome 5q and 7q.

A review of 431 cases of secondary leukemia found 16 nonrandom chromosomal changes involving chromosomes 3, 5, 7, 8, 9, 11, 14, 17, and 21. These changes were dependent on the type of primary disease, previous therapy, age, and gender [64]. In another single-institution study consisting of 63 patients, additional abnormalities involving chromosomes 1, 4, 5, 7, 12, 14, and 18 occurred, with significantly increased frequency of these changes in t-AML as compared to *de novo* AML [34]. Abnormalities in chromosome 17, especially translocations involving bands 17p11-p13 and 17q21, occasionally are observed in t-AML, for example, t(15;17)(q22;q11–21) [65, 66]. Other chromosomes often reported to be abnormal in t-MDS/t-AML are chromosomes 21 and 11, particularly involving balanced translocations of chromosome bands 11q23 and 21q22 in t-AML [i.e., t(4;11), t(6;11), t(9;11), t(11;19), t(3;21), and t(8;21)] [52, 59, 63, 64]. These translocations are associated with previous

therapy targeting DNA-topoisomerase II, primarily the epipodophyllotoxins and the anthracyclines. The 11q23 reciprocal translocations and interstitial deletions structurally interrupt a small region of the *MLL* (also known as *HRX, ALL-1, HTRX1*) gene that codes for a human homolog of the *Drosophila trithorax* gene [67, 68]. A fragment of the *MLL* gene translocates to more than 200 other chromosomal regions, resulting in the creation of a fusion protein with the partner gene [69].

The t(9;11) that results in a fusion between *MLL* and *AF9* is a recurring chromosomal translocation in *de novo* AML and is one of the most common recurring chromosome translocations detected in about 50% of t-AML patients who have a *MLL* translocation [70]. In addition, involvement of the *AF9* gene in the development of t-AML is linked to the treatment with topoisomerase inhibitors [6]. Interestingly, the unbalanced rearrangements of the same two bands, 11q23 and 21q22, were most often associated with therapy with alkylating agents alone or in combination with radiation therapy [59]. In Chinese patients treated for psoriasis with bimolane, t(15;17) has been frequently reported. Also, therapy with doxorubicin has been associated with an increased incidence of t-AML with balanced translocations at chromosome band 21q22, in particular t(3;21) [52, 64]. In addition to balanced translocations involving chromosome bands 11q23 and 21q22, other balanced aberrations such as inv (16), t(8;16), t(15;17), and t(6;9) have been observed in t-AML after previous therapy with drugs targeting DNA topoisomerase II (Table 22.2) [52, 63].

In a study of 491 t-MDS/t-AML patients with at least one balanced translocation, Rowley and Olney reported that 149 of the patients were positive for the 11q23 translocation (30.3%), followed by the 21q22 rearrangement seen in 15%, inv (16) in 9%, and t(15;17) in 8% of the patients [70]. Interestingly, no significant difference occurred in the gender distribution of patients within the subgroups, and patients in the 11q23 subgroup were of the youngest age at their primary and secondary diagnosis. Moreover, the translocation 11q23, inv (16), and t(15;17) subgroups had the shortest latency, with a median latency of 25.9 months for translocation 11q23, 22.0 months for inv (16), and 28.9 months for t(15;17) [70].

Table 22.2 Thirty-eight HSC gene signatures predicting development of t-MDS/AML

Thirty-eight genes differentially expressed in CD34+ HSC of t-MDS/AML patients
NR4A2, FOS, EGR1, CARD6, PEX11B, EGR3, EGR4, MRPL15, SLC7111, REEP1, FOSB, GOLGA5, ACTL6A,
GOLPH3L, CCDC99, SMAD7, SHMT2, LRPPRC, CDCA4, PDIA4, GOT1, RTN3, KLF2, JUN, STK17B, PSMC2,
LRBA, XPOT, ZYG11B, ZNF137, GEM, PGRMC2, ARL6IP6, SLC2A3P1, NR4A3, RGS2, NROP3, SLC26A2

Chromosome studies have shown that when t-MDS becomes clinically diagnosable, the preleukemic clone represents a majority of the hematopoietic cells [7, 34, 49]. Additional chromosomal abnormalities occur in the original abnormal clone in 60–70% of cases as the disease evolves to frank leukemia [71, 72]. Karyotypic evolution usually involves further deletions or losses of chromosomes and a change to a lower modal chromosome number; rarely, the evolution is associated with a gain of chromosome 8. Evidence suggests that t-MDS patients who have a mixture of karyotypically normal and abnormal cells (AN) survive longer than those who have only abnormal cells (AA) [73]. Most individuals who are AN in the preleukemic phase become AA as the disease progresses [34]. Notably, Rowley and Olney observed in their study that patients presenting with a t-MDS had significantly more frequent abnormalities of chromosomes 5 and/or 7 (49%) than did patients presenting with a t-AML (16%), and that this subgroup also presented with the highest percentage of complex karyotypes (45% vs. ca. 20% for both 1 and 2 aberrations) [70].

Genetics of Therapy-Related AML

Patients who develop t-AML may be predisposed to develop AML because of defects in DNA repair or increased susceptibility to accumulation of genetic mutations [74]. Candidate single-nucleotide polymorphisms associated with either drug metabolism or DNA repair enzymes have been identified as a mechanism by which a subset of t-AML may develop [75]. The commonly found germline variants in t-AML patients are *NQ01*, glutathione S-transferase family of enzymes, *BRCA1/2*, *TP53*, and *MDM2* [76–78]. Li-Fraumeni syndrome and Fanconi anemia also predispose to acute leukemia [79]. Tumor protein 53 (*TP53*), *RUNX1*, V-Ki-ras2 Kirsten rat sarcoma viral oncogene homolog (*KRAS*), and neuroblastoma RAS viral (v-ras) oncogene homolog (*NRAS*) mutations are known mutations in the development of t-MDS/AML [80].

To understand the pathogenetic mechanisms underlying t-MDS/AML, Li et al. performed a prospective case-control study with patients undergoing autologous hematopoietic stem cell transplant for lymphoma. In the study, gene expression in CD34+ hematopoietic stem and progenitor cells (HSC) from patients who developed t-MDS/AML after autologous hematopoietic cell transplantation (autoHSCT) for lymphoma (n = 30) was compared with gene expression in CD34+ cells of control group. The authors demonstrated that the expression pattern of 38 genes was different long before the development of t-MDS/AML in the case group, and that this gene signature was involved in mitochondrial function, metabolism, and hematopoietic regulation in peripheral blood stem cells (PBSC) that could distinguish patients who developed t-MDS/AML post-autoHSCT from those who did not [81].

Table 22.3 Mutational profiles of therapy-related acute myeloid leukemia versus *de novo* AML [82, 83]

Group	More frequent in t-AML	Similar frequency in t-AML and *de novo* AML	More frequent in *de novo* AML
Genes mutated	*TP53*, ATP-binding cassette subfamily genes, *PTPN11*	*STAG2, DNMT3A, NRAS, KRAS, IDH1, IDH2, U2AF1, KIT, KHD1, PKDL2, TET2, RUNX1*	*FLT3, NPM1*

Another study assessed the bone marrow or peripheral blood samples of 70 t-MDS/AML (including 42 t-AML) patients using a next-generation sequencing of 53 targeted genes. The mutation profile of t-AML was different from those of 428 *de novo* MDS/AML patients [82]. TP53 was mutated at a significantly higher rate in t-AML than *de novo* AML (35.7% vs. 12.8%, $p = 0.002$). *PTPN11* mutations were observed in 11.9% of t-AML patients compared with 2.1% in *de novo* AML patients ($p = 0.008$). Mutations of *NPM1* and *FLT3* only occurred in 2.5% and 7.1% of t-AML patients, respectively, which was significantly lower than *de novo* AML patients (21.7% and 16.4%, respectively) (Table 22.3). Analysis of clonal evolution showed that *TP53* mutation often occurs early in the pathogenesis of t-AML, and mutations of other genes may provide a further evolution to t-AML [83].

Lindsley et al. reported the genetics of 101 t-AML patients [84]. The goal of the study was to find a distinct mutation profile of t-AML compared with secondary or *de novo* AML with comprehensive sequencing. Samples obtained from patients prior to treatment were analyzed for mutations in 82 genes and the results were compared to the genetic profiles in The Cancer Genome Atlas of *de novo* AML. The comparative analysis demonstrated three mutually exclusive patterns of mutations that were noted in the t-AML cohort. The first group had *TP53* mutations. The second group had mutations which were commonly associated with secondary AML. These "secondary" mutations at cohort included spliceosome genes (*SRSF2, SF3B1, U2AF1, ZRSR2*), chromatin remodeling genes (*ASXL1, EZH2, BCOR*), and cohesion gene (*STAG2*). The third group included those with *de novo*-type mutations (*NPM1*, CBF rearrangements, and MLL rearrangements). However, the authors did not find unique genetic profiles, associated with chemotherapy exposure other than the established link between exposure to topoisomerase II inhibitors and MLL rearrangements. The CR rate for t-AML patients with *de novo*-type mutations was less than in *de novo* AML patients with the same mutations, but t-AML with secondary-type mutations or *TP53* mutations had remission rates similar to the older *de novo* AML group with the same "secondary"-type mutations. But the t-AML cohort required more cycles of induction therapy for CR. Taken together, prior chemotherapy exposure may not produce a unique "therapy-related" genetic profile, but genetic profiles may help predict outcomes of t-AML patients [84].

Next-generation sequencing could identify mutations in the leukemic transformation of Severe Congenital Neutropenia (SCN). Mutations in *CSF3R*-T618I, *RUNX1*, and *ASXL1* were found only in the MDS/AML phase of SCN [85]. Another study revealed that 64.5% of patients of the study population had mutations in *RUNX1* and the mutation occurred in clones with earlier acquired *CSF3R* mutations [86]. A sequential analysis at stages prior to leukemia development demonstrated that the *RUNX1* mutations are late events in the AML development of SCN. The other mutations associated with leukemic transformation were *ASXL1*, *SUZ12*, and *EP300* in less frequent rate.

Several groups have tried to identify the major genetic mutations in progression of MPN to AML by performing genotypic analyses of AML cells evolved from MPN [87–92]. The process of AML transformation is considered to arise from additional mutations outside of the JAK-STAT pathway, which is supported by the findings that the canonical *JAK2*V617F mutation has not been correlated with leukemic transformation. Furthermore, this mutation can be absent in the leukemic clone [93]. In these analyses, mutations affecting epigenetic regulators and transcriptional factors (*ASXL1, TET2, EZH2, IDH1/IDH2, IKZF1*), splicing factors (*SRSF2, SF3B1, ZRSR2, U2AF1*), and *TP53* mutations were frequently observed in MPN-AML cells compared to MPN cells. While only 1.9% of PV patients had an *IDH1/2* mutation, MPN-AML patients had a high frequency (21.6%) of this mutation [94]. Among 22 patients with post-MPN AML, 45.5% of the patients had a P53-related defect. In a study of 29 post-MPN AML samples (including 162 chronic-phase PV) using SNP arrays, changes of chromosomes 1q, 7q, 5q, 6p, 7p, 19q, 22q, and 3q were associated with post-MPN AML [88].

Clinical Presentation and Diagnosis

No specific clinical presentation demarks t-AML, but most patients have symptoms similar to patients with *de novo* AML including cytopenias (i.e., anemia, neutropenia, and thrombocytopenia) associated with easy fatigue, generalized malaise, infections, and/or hemorrhagic symptoms as easy bruising, nose/gingival bleeding, menorrhagia, or petechiae. Patients may have clinical manifestations of hepatomegaly, splenomegaly, lymphadenopathy, gingival hypertrophy, skin infiltration, and neurological abnormalities.

A preleukemic or myelodysplastic phase occurs in over 70% of patients in whom AML develops following chemotherapy and/or radiation therapy for another disease [7, 27, 49–51, 95–98], whereas about 20% of patients with *de novo*

AML have a similar preleukemic phase. Indeed, the data suggest that a preleukemic period can be observed in nearly all patients with t-AML, when these patients are monitored closely. Exceptions are those individuals in whom t-AML develops after therapy with epipodophyllotoxins (VP16 and VM26) or other DNA-topoisomerase II inhibitors. In these patients, t-AML often develops with no preleukemic phase [16, 17]. The mean duration of the preleukemic phase is 11.2 months in typical t-AML. The preleukemic phase in individuals with *de novo* MDS who go on to develop AML is similar, about 14 months (Table 22.1) [95, 96, 98].

Prodromal symptoms of the emergence of t-AML may be similar. However, when a patient who has received cytotoxic agents has these symptoms, the appropriate workup to rule out t-AML should be done. The diagnostic evaluation includes a comprehensive medical history and physical examination with detailed information of exposure to cytotoxic agents (time, duration, cumulative doses). In addition, patient's age, comorbidities, performance status, organ dysfunction, and remission status of the primary disease are important for establishing management plan. A detailed family history is essential to rule out hereditary cancer syndrome. Peripheral blood smear is an important laboratory test to rule out dysplastic changes in myeloid cells. Complete blood differential counts, and metabolic panel, as well as lactate dehydrogenase and uric acid level are required for initial laboratory tests. The diagnosis of t-AML is eventually made when evaluation of the peripheral blood and bone marrow demonstrates circulating myeloblasts in peripheral blood and/or more than 20% of myeloblasts in bone marrow. This may be buttressed by typical immunophenotypic and cytogenetic changes.

The clinical manifestation of the preleukemic phase of t-AML is marked by ineffective hematopoiesis. The bone marrow morphology is characterized by trilineage dysplasia. The degree of dysplasia is usually very prominent. Interestingly, the RAEB and RAEB-t subgroups are more frequently linked to t-MDS (73%) than in *de novo* MDS (53%) [99]. Prominent abnormalities are observed in the red blood cells and their precursors. Most patients show decreased red cell production with low reticulocyte counts [27]. Oval macrocytosis and nucleated red cells are often the earliest recognizable changes observed in the peripheral blood in the preleukemic phase [100]. Macrocytosis after therapy for Hodgkin's disease was retrospectively found to be associated with a high risk of the development of leukemia [101]. Mild neutropenia is present in 75% of the individuals [27]. Neutrophils may be poorly granulated, and their nuclei can be hyposegmented (pseudo-Pelger-Hüet anomaly) [102]. Thrombocytopenia occurs in approximately 60% of patients [27], and they may be abnormally large and degranulated. Both the neutrophils and platelets can have a variety of qualitative defects. The bone marrow is often hypercellular, although hypo- and normocellular marrow can occur. Erythroid hyperplasia, megaloblastoid features, and occasionally ringed sideroblasts dominate the marrow picture [102–104]. Abnormalities of the marrow granulocytic and megakaryocytic series are usually more subtle. Micromegakaryocytes may be seen, particularly with monosomy 7. The percentage of immature granulocytic and megakaryocytic cells may be increased. The primary and specific granules of the granulocyte precursors occasionally are either deficient or abnormally large. Marrow fibrosis often is present during the preleukemic phase.

In summary, the development of unexplained pancytopenia and the finding of karyotypic abnormalities in the marrow cells of patients who received chemotherapy and/or radiation therapy for another disease are pathognomonic of preleukemia. Evolution to overt leukemia is universal if the preleukemic individual survives the complications of hemorrhage and infection. T-MDS can be viewed as an early phase of t-AML in which the malignant hematopoietic clone is established and becomes predominant.

Clinical manifestations of individuals with t-AML are typical of bone marrow failure, and their clinical course is rapidly fatal often from complications of bleeding and infection. The bone marrow morphology of t-AML has been difficult to classify according to FAB criteria for AML, as most of the leukemias demonstrate trilineage involvement and appear to bridge several subtypes. Nevertheless, the blast cells of patients with t-AML most often are myeloblastic in appearance according to AML without maturation or AML with minimal differentiation in agreement with the 2008 WHO classification. A lower frequency of acute monocytic forms of leukemia has been reported in several studies as compared to *de novo* AML [7, 105].

Auer rods are rarely observed in the blast cells in t-AML, but are seen in blast cells of 35% of patients with *de novo* AML. Many of the blast cells in t-AML lack myeloperoxidase and other granulocyte-specific enzymes. In one series, only one of ten patients with secondary AML had more than 10% peroxidase-positive blast cells compared with nearly 100% peroxidase-positive blast cells in 95% of patients with *de novo* AML [105]. In addition, less than 20% of the t-AML patients have either greater than or equal to 10% naphthol ASD chloroacetate esterase-positive blast cells compared with 47% of patients with *de novo* AML. These histochemical data suggest that the leukemic cells from secondary leukemia patients are blocked at an earlier stage of differentiation than the leukemic cells from most *de novo* AML patients.

A patient may develop t-AML after cytotoxic treatment for a *de novo* myeloid neoplasm. T-AML secondary to *de novo* myeloid neoplasm may be identified by performing cytogenetic testing and immunophenotype evaluation at

Table 22.4 Initial evaluation of a therapy-related acute myelogenous leukemia

History and physical examination	History of cytotoxic agent: cumulative doses Disease status of primary cancer Performance status Family history of cancer
Complete blood cell count, differential count	
Review of peripheral blood smear	
Serum chemistries	Liver/kidney function, LDH, uric acid
Bone marrow aspirate and biopsy	Immunophenotyping and cytogenetic analysis
HLA typing	
Organ function tests	Echocardiogram/MUGA for ejection fraction Pulmonary function tests

apparent t-AML development and by comparing with those at the time of primary disease diagnosis. The emergence of a distinctly different karyotype suggests, but does not prove, a therapy-related AML, rather than recurrence of the original leukemic clone.

For patients with good performance status, information about siblings is useful for establishing a management plan. HLA typing of patient can be done on final diagnosis and the identification of potential stem cell donor is the first step for matched related donor allogeneic transplant. To evaluate reserved organ function, echocardiogram, and pulmonary function tests is also required. Computed tomography/MRI or PET imaging can give information about the status of primary disease (Table 22.4).

Treatment

Effective treatment options for t-AML are often not available. The efficacy of various therapeutic modalities of t-AML has been difficult to assess because the number of reported cases is small. In addition, data for t-AML have been reported together with secondary leukemias following other hematological disorders such as MPN or *de novo* MDS, making the evaluation difficult. Daunorubicin in combination with cytarabine remains the standard induction chemotherapy combination for patients with AML for the last decades. All studies to date have shown that response rates, and OS, are significantly lower in whole t-AML patients compared with *de novo* AML. Complete remission rates in t-AML patients are reported in 40% of patients with median survivals of 6–8 months [106]. The treatment of patients with t-AML is a clinical challenge for multiple reasons. The patients have a greater number of comorbidities, decreased organ reserve from previous therapy or primary disease, and a higher incidence of unfavorable cytogenetic changes. The key prognostic factors in t-AML are patient age, performance status, and karyotype.

For all t-AML patients, the performance status is the first determinant for establishing a treatment plan. All medically fit patients should have HLA typing at initial diagnosis. Supportive care would be appropriate for patients with a poor performance status (ECOG PS >2) at initial diagnosis. Various attempts to improve survival in these patients have failed to change the course of the disease, with deaths due to infection, bleeding, or progression of the acute leukemia. Supportive therapy is, therefore, an important aspect of the medical care of these patients. No significant differences in survival have been shown between those patients who received chemotherapy and supportive care and those who received supportive care alone. Thus, supportive therapy with transfusions of red blood cells and platelets for symptomatic anemia or bleeding complications, or both, is often necessary as well as the treatment with antibiotics for infections. The goal of therapy in these individuals should be to maintain an acceptable quality of life. Clearly, innovative and radically novel approaches to this syndrome are required if these patients are to be cured.

Studies have suggested that no significant differences exist in clinical outcome between the t-AML and *de novo* AML in the same cytogenetic group. The therapy-related AML with favorable cytogenetic findings, such as t(8;21), t(15;17), and inv. (16), have a complete response rate, essentially the same as *de novo* AML with the same karyotype [63, 107, 108]. Similarly, patients who have secondary AML with unfavorable cytogenetic findings such as deletion of chromosomes 5 or 7 do poorly, similar to *de novo* AML individuals with the same abnormality. Therefore, though more single or complex clonal cytogenetic abnormalities are found in t-AML patients than *de novo* AML patients [3], the prognostic significance of karyotype in t-AML is similar to that in *de novo* AML.

No single form of post-remission therapy has been shown to be superior for t-AML. Post-remission therapy with high-dose cytarabine probably is appropriate in patients with favorable cytogenetic findings except for those t-APL patients with t(15;17). In contrast, because of their extremely poor outcome, patients with unfavorable cytogenetic findings should be encouraged to enter clinical trials. As part of the discussion of treatment options, t-AML patients with an extremely poor prognosis should probably be offered the spectrum of treatments from supportive care alone to intensive chemotherapy either with or without allogeneic hematopoietic stem cell transplantation.

Therapy-Related AML Patients with Favorable Cytogenetics

Therapy-related AML is a heterogeneous disease and cytogenetic profile remains prognostically relevant. Patients with t-AML and favorable cytogenetics including t(15;17), inv.(16), t(16;16), and t(8;21) generally have superior outcomes among patients with t-AML.

A European study identified 106 cases of t-APL in patients who received cytotoxic chemotherapy for breast cancer, non-Hodgkin's lymphoma, and other solid tumors over a period of 10 years [109]. These t-APL patients had a short latency time (2–3 years), and exposure to topoisomerase II inhibitors or prior radiation therapy, and shared similar clinical characteristics with *de novo* APL [110, 111]. Yin et al. demonstrated frequent dyserythropoiesis, dysmegakaryopoiesis, FLT3 mutation (43%), and frequent additional cytogenetic abnormalities (60%) in their report of 17 t-APL patients [112]. Mounting evidence supports the practice of treating t-AML with t(15;17) as *de novo* disease, even when accompanied by other karyotype abnormalities [110]. They have a good response to all-trans retinoic acid (ATRA) therapy. Induction response rates appear to be equivalent to *de novo* APL, but induction death was more common and was attributed to impaired physiologic reserves from prior therapy. Therefore, t-APL is currently treated as *de novo* APL. With the use of ATRA and arsenic trioxide (ATO) in up-front therapy, anthracyclines can be eliminated for low-risk APL, which would be particularly beneficial for patients with t-APL who have had a prior anthracycline therapy for their primary malignancy [113].

T-AML with t(8;21) is not a common type of t-AML. A review article noted 26 cases and concluded that these patients had very similar hematological characteristics and treatment response as *de novo* AML with t(8;21) [63]. The 2002 international workshop studied 72 cases of t-AML with 21q22 (RUNX1) rearrangement and found that 44 of these cases were t(8;21) [114]. In the study, patients with t(8;21) rearrangement had a more favorable outcome than patients with other rearrangements involving 21q22. Gustafson et al. observed 13 patients with t-AML having t(8;21) karyotype in a single institute and compared them to 38 patients with *de novo* AML with t(8;21) and found that patients with therapy-related t(8;21) AML were older, and had a higher frequency of *KIT* 816D mutations, and an inferior OS than their *de novo* counterparts [115]. Krauth et al. showed high frequencies of additional cytogenetic and molecular lesions in AML with t(8;21) [116]. Mutations in RAS pathway, *KIT* and *ASXL1* mutations, were the most frequent additional mutations in the study, and mutations in *KIT* D816 and *ASXL1* were strongly associated with adverse outcomes. At the chromosomal level, −Y appeared to be associated with a good prognosis whereas trisomy 8 had an inferior prognosis. In a large series of t(8;21), 22 t-AML patients showed no differences in secondary molecular genetic events from 117 *de novo* AML [116]. However, a study showed that the treatment outcomes of t(8;21) t-AML were inferior to those of *de novo* t(8;21) AML, possibly because the t-AML cohort was older and some patients had active primary cancer.

T-AML with inv (16) was often associated with prior therapy with topoisomerase II inhibitors [117]. Response rates to intensive chemotherapy in this study were comparable to those with *de novo* disease. However, t-AML with inv (16) showed a significantly shorter event-free survival than *de novo* AML. In general, secondary chromosomal aberrations as well as gene mutations are very frequent in AML with inv (16); 80–90% patients with inv (16) AML have at least one mutation involving *NRAS, KRAS, KIT*, or *FLT* [118–120]. In the German-Austrian AML Study Group (AMLSG) study, 12 patients out of 176 cases (7%) were considered to be therapy related and the secondary chromosomal abnormalities/mutations were not significantly different from *de novo* AML [120], suggesting that the additional mutation is not the reason for shorter event-free survival in t-AML patients after intensive chemotherapy.

In summary, t-AML with favorable cytogenetics shows similar response rate to their *de novo* counterpart when receiving a conventional AML treatment. However, compared to *de novo* counterparts, t-AML with favorable cytogenetics is associated with an inferior survival. This may relate to several factors such as the status of primary disease, toxicity from prior therapy, and additional genetic mutations. Considering that additional mutations in t-AML patients with favorable karyotypes may result in poor prognosis, comprehensive genetic tests may confer an appropriate decision making, especially in patients cured of the primary malignancy and who are good candidates for allogeneic HSCT.

Non-transplant Therapeutic Options for t-AML

Few retrospective studies have evaluated the efficacy of standard chemotherapy for t-AML. The German AML Cooperative Group analyzed outcomes after remission induction chemotherapy for 1511 *de novo* AML and 121 t-AML patients [121]. The study demonstrated that the survival of unfavorable and intermediate cytogenetic risk groups of t-AML was similar with the same risk groups of *de novo* AML (6 months vs. 7 months for unfavorable, 12 months vs. 16 months for intermediate-risk group, respectively). Another study, the German–Austrian AL Study Group assessed the clinical outcomes of 200 t-AML patients treated between 1993 and 2008 [3]. The survival of t-AML patients was compared with 2653 *de novo* AML patients. Although response rates to induction chemotherapy were similar, OS for t-AML patients was inferior to *de novo* AML patients.

In further analysis, patients less than 60 years old showed similar relapse rates, but their death in CR was greater, suggesting the higher toxicity of induction and post-remission therapy in this cohort. Patients older than 60 years had higher relapse rates, possibly due to lower intensity treatments, resulting in inferior survival. A retrospective study of 118 t-AML after treatment of breast cancer showed no significant difference in median OS compared with *de novo* AML (8.7 months vs. 10.2 months; $p = 0.17$) [122]. Multivariate analysis revealed cytogenetics, baseline white blood cell counts, age, and performance status as predictive factors for OS of t-AML patients.

In a prospective study of t-MDS/AML, 32 t-MDS/AML patients were treated with high-dose cytarabine and mitoxantrone induction followed by hematopoietic stem cell transplant [101]. A remarkable complete response rate of 66% was achieved. Thirteen patients who achieved CR were eventually treated with AlloHSCT for consolidation and the survival of the patients was 29% in 3 years. These studies show that patients with t-AML can achieve a comparable response with standard induction chemotherapy, and that cumulative toxicity/reserved function from prior therapy limit tolerance to induction and post-remission therapy.

T-AML patients have a higher risk of organ dysfunction due to chemotherapy and radiation-induced parenchymal and vascular toxicity, or primary malignancy. Even those with seemingly adequate organ reserves may have increased toxicity during t-AML therapy. Therefore, earlier diagnosis and treatment with less toxic therapy, while aggressively exploring transplant options, may be another critical factor in the trial of new therapeutics for t-AML. Emerging therapeutics in this area has focused on several approaches. These include novel delivery of chemotherapy as well as newer DNA-damaging agents delivered through antibody-drug conjugates, use of hypomethylating agents, and molecularly directed small molecules against specific mutations commonly occurring in t-AML.

CPX-351 is a liposomal formulation of daunorubicin and cytarabine at a fixed ratio of 5:1. The combination of these medications was developed based on in vitro data that demonstrated a synergistic effect of these two agents at the 5:1 ratio [123]. In a randomized phase II trial, CPX-351 was compared with standard daunorubicin/cytarabine in untreated patients older than the age of 60 years [124]. In a subset of secondary AML patients ($n = 52$), which included t-AML and AML evolving from myelodysplastic syndrome, patients treated with CPX-351 demonstrated a better OS (hazard ratio = 0.46, $p = 0.01$) at 24 months. Though the recovery from cytopenias was slower after CPX-351, the infection-related deaths (3.5% vs. 7.3%) or 60-day mortality (4.7% vs. 14.6%) was less than the conventional daunorubicin/cytarabine chemotherapy group. These data suggested a clinical benefit with CPX-351 in t-AML with better efficacy and tolerability.

A second approach to improving cytotoxic therapy for AML takes advantage of newer antibody-drug conjugate (ADC) technology. CD33 is a surface receptor found on more than 95% of AML cells except acute megakaryocytic leukemia. It has been a target for antibody-directed therapy. The treatment with a conjugated antibody targeting CD33 (gemtuzumab ozogamicin) as a single agent [125], and in combination with chemotherapy in untreated patients and those with relapsed AML, demonstrated clinical efficacy [126]. However, gemtuzumab ozogamicin failed to show the effectiveness in combination with standard daunorubicin/cytarabine regimen in high-risk AML patients [127]. A new ADC targeting CD33, SGN-CD33A, was developed using a novel antibody drug linkage system to a fully humanized anti-CD33 antibody. In contrast to gemtuzumab ozogamicin, SGN-CD33A exhibited a potent cytotoxicity against p53-mutated AML cells and leukemic cells with multidrug resistance-mediated drug efflux phenotypes in preclinical studies [128]. Therefore, careful clinical trials with this monoclonal antibody conjugate are appropriate for t-AML patients.

Since many cases of t-AML evolved from a preleukemic phase after being exposed to chemotherapeutics/radiation, hypomethylating agents have been evaluated as an alternative to traditional induction therapy [129]. Both azacitidine and decitabine are effective and well tolerated but the efficacy compared with cytotoxic chemotherapy is still under investigation. In a retrospective study conducted by Quintas-Cardama et al., 671 AML patients, older than 65 years, were treated with a hypomethylating agent and had a similar median survival rates with cytotoxic chemotherapy (6.5 months with hypomethylating agent and 6.7 months with chemotherapy, respectively) [130]. Moreover, a similar CR rate was observed in the subset of poor-risk cytogenetics patients carrying −5 and/or −7 (26% with hypomethylating agent vs. 28% with chemotherapy). Another retrospective study of 48 t-MDS/AML patients treated with hypomethylating agent showed 42% overall response rate including a complete response rate of 21% in a subset of patients with favorable cytogenetics, which is comparable with prospective hypomethylating agent studies for t-MDS group [131]. A phase 2 clinical trial, E1905 North American Leukemia Intergroup, studied 47 patients including 18 t-AML patients. A good response to azacitidine occurred with 46% complete hematologic response and 13 months of median OS [132]. Multivariate analyses comparing the t-MN patient with *de novo* MDS/AML patients treated with the same protocol showed no significant difference in complete hematologic response rate, and overall response rate between the two groups. However, another study of 54 t-MN patients (including 12 t-AML patients) treated with azacitidine demonstrated shorter 2-year OS (14%) compared with *de novo* MDS/AML patients (33.9%), though multivariate analysis showed that the survival was dependent on cytogenetic changes, not etiology of the AML [133].

Hypomethylating agents are frequently prescribed as an alternative to traditional AML induction chemotherapy for frail patients. They can support *de novo* and t-MDS/AML patients in order to receive a transplantation with less toxicity, and may be a safer option for low-blast-count t-AML. Response rates were equivalent to standard AML induction therapy in this population [131, 134], enhancing the likelihood of successful transplantations. A new hypomethylating agent, SGI-110, a metabolite of decitabine, is in clinical trials for treatment of MDS and AML [135].

An additional epigenetic modulator is the class of histone deacetylation (HDAC) inhibitors. They are often included in a combination regimen with a hypomethylating agent for MDS and AML patients. Valproic acid, vorinostat, pracinostat, and mocetinostat are the HDAC inhibitors being used in clinical trials in combination with hypomethylating agents [136–139].

P53 mutations and *MLL* rearrangements often occur in t-AML [140, 141]. Two agents targeting these mutations are in the drug pipeline. EPZ-5676 is a potent inhibitor of Dot1L, a histone methyl transferase which interacts with MLL oncogenic fusion protein products. In cell lines and in rat xenograft studies, EPZ-5676 significantly caused cell death and regression of MLL-rearranged leukemias [142]. It is currently in clinical trial in pediatric leukemias with MLL translocations.

Volasertib is an inhibitor of polo-like kinase (PLK). Preclinical studies demonstrated that p53-mutated cancer cells were more susceptible to PLK inhibition than p53 wild-type cancer cells [143]. In a randomized phase 2 study in untreated elderly patients with AML, volasertib, in combination with low-dose cytarabine, demonstrated a higher remission rate and improved survival compared with cytarabine alone, although median survival rates were still <1 year [144].

Hematopoietic Stem Cell Transplant for t-AML

Treatment of t-AML with conventional therapy is associated with a poor outcome. Response rate for t-AML induction therapy appears to be roughly equivalent to *de novo* AML when compared within their respective intermediate- and unfavorable-risk cytogenetic categories, but the responses on average are less durable, thereby justifying the use of transplantation in these patients.

A retrospective study of 545 t-AML patients transplanted between 1990 and 2004 found an OS of 22% at 5 years [145]. Inferior outcomes were associated with age greater than 35 years, poor-risk cytogenetics, uncontrolled disease, and use of a non-sibling-related or mismatched unrelated donor. Use of reduced-intensity chemotherapy (RIC) regimens did not decrease treatment-related mortality (TRM), which approached 50% at 5 years, but many of these patients had received a prior autologous transplant.

The European Group for Blood and Marrow Transplantation Group also reported on 461 t-MDS/AML patients, and noted an adverse impact of abnormal cytogenetics, age greater than 40 years, and uncontrolled disease [146]. Three-year relapse-free survival and OS rates were 33% and 35%, respectively. In contrast, a study of 24 breast cancer t-MN patients who underwent allogeneic stem cell transplantation for consolidation had nearly identical clinical results as female *de novo* MDS/AML patients regardless of cytogenetics [147]. In general, these studies show that transplantation can be used successfully in a fraction of t-AML patients, but it is clearly less effective than when used for *de novo* AML patients. A busulfan/cyclophosphamide conditioning regimen appears to offer one of the best 5-year relapse-free survival (43%) and lowest non-relapse mortality (28%). Relapse rates are lower with unrelated donor transplants [145, 146, 148]. After accounting for cytogenetic classification, t-AML patients have a similar outcome as *de novo* AML [149].

The Italian Network reported survival for transplant recipients of 58.8 months compared with 12.1 months for the non-transplant cohort [150]. A similar benefit was seen when the German Hodgkin Study Group reported clinical outcomes of 106 patients with t-MN after therapy for Hodgkin's lymphoma. Although the non-transplant median survival was dismal (7.2 months), the median survival for the transplanted t-MN had not been reached after a median follow-up of 41 months [151]. The survival of the patients after hematopoietic stem cell transplant at 2 years was 47% vs. 15% for the non-transplant group ($p = 0.03$). Although alloHSCT can provide a chance of long-term survival and cure in selected subgroups of patients with t-AML, major limitations of alloHSCT are availability of a donor and patients' age. Alternative treatment strategies including haploidentical donor alloHSCT or nonmyeloablative HSCT, especially for older patients, should be explored for t-AML patients.

Conclusion

Therapy-related AML (t-AML) is a recognizable subgroup of AML. Alkylating agents used in primary diseases are the most frequent etiology of t-AML. The disease arises from a series of mutations in hematopoietic stem cells, and these DNA changes provide a growth advantage to the progeny of the transformed cells. The abnormal clone of cells usually has a hypodiploid modal number of chromosomes and a deletion of part or all of chromosome 5 and/or 7. T-AML remains one of the most difficult subtypes of AML to treat. Once a patient who was treated with cytotoxic agents develops cytopenias, hematopoietic cell morphologic examination, immunophenotying, and cytogenetics should be done to detect t-AML in its early phase. The patients with t-AML have more comorbidities, decreased organ reserve, and a higher incidence of unfavorable cytogenetic phenotype than

de novo AML. The key prognostic factors in t-AML are patient age, performance status, and karyotype. As *de novo* AML, t-AML patients can be stratified based on genetics. The performance status is the first determinant for establishing a treatment plan. Supportive care at initial diagnosis would be appropriate for patients with a poor performance status (ECOG PS >2). All medically fit patients should have HLA typing at initial diagnosis. The conventional cytotoxic chemotherapy or hypomethylating agents are being used as an initial therapy. For patients in complete remission, allogeneic transplantation is the best therapeutic modality for long-term survival for the younger patients. Emerging therapeutics for AML has focused on reduced toxicity, higher efficacy, and specificity. These include novel delivery of chemotherapy in liposome as well as newer DNA-damaging agents delivered through antibody-drug conjugates, use of hypomethylating agents, and molecularly directed small molecules against specific mutations commonly occurring in t-AML.

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The Myelodysplastic Syndromes

23

Kenneth Miller and Monika Pilichowska

Introduction

The myelodysplastic syndromes (MDS) are a heterogeneous group of clonal stem cell disorders characterized by impaired, and/or ineffective, proliferation and maturation of hematopoietic progenitor cells resulting in symptomatic anemia, leukopenia, or thrombocytopenia. The clinical course is very variable, ranging from a chronic, stable, mildly symptomatic disorder to a malignancy that rapidly progresses to AML. Morphological and functional cellular abnormalities involving one or more cell lines are common resulting in infections and/or bleeding. The complications from MDS and its treatment remain the major cause of morbidity and mortality. MDS shares many clinical, cytogenetic, and laboratory features with aplastic anemia, hypoplastic anemias, the myeloproliferative neoplasms, and the acute leukemias. The updated WHO classification attempts to define the diagnosis of MDS and separate it from the myeloproliferative neoplasms, reactive and secondary causes of hypoproliferative disorders and the acute leukemias. Patients with MDS may present with clinical and laboratory features suggestive of a reactive, autoimmune or other malignant stem cell disorder. Therefore, in many instances, the diagnosis of MDS is based on the exclusion of other disorders associated with dysplasia and impaired or ineffective hematopoiesis.

MDS is a progressive clonal disorder and the diagnostic studies and the initial evaluations are similar to those used to define other neoplastic and non-neoplastic stem cell disorders. However, the clinical course, prognosis, and treatment approach for a patient with a MDS is different from the other neoplastic stem cell disorders. The bone marrow and peripheral blood abnormalities in MDS can be subtle and require the cooperative efforts of pathologists, cytogeneticists, and clinicians to diagnosis, classify and define prognosis. MDS is one of most common hematologic malignancies in older populations and treatments remain controversial and limited for many patients. Treatments for many patients should be individualized and tailored to the MDS subtype, patient's age, comorbidities, and other prognostic variables.

Myelodysplasia, derived from the Greek and meaning morphological abnormality of the bone marrow, is not a new disease but it has only recently been assigned as a separate category in the classification of malignant hematopoietic disorders based on specific diagnostic criteria. The initial reports identified elderly patients with progressive cytopenias, morphologically abnormal cells, and a propensity to progress to an acute leukemia. Terms such as smoldering leukemia, or preleukemia were used to describe this condition, reflecting that patients with MDS did not meet the usual criteria of acute leukemia but presented with a syndrome which included a hypercellular bone marrow with increased blast forms and dysplastic changes in one or more cell lines. In MDS, as in the myeloproliferative neoplasms and acute leukemias an oncogenic transforming event occurs at the level of the myeloid or pluripotential stem cell. Cytogenetic studies were first to identify the genetic alterations in MDS and are important in establishing the diagnosis and prognosis in MDS. More recently numerous specific gene mutations have been identified in MDS associated genes that may define prognosis and impact treatment. While many patients with MDS have features suggestive of an early, smoldering leukemia, and rapidly evolve into one of the myeloid leukemias, most patients do not evolve into AML but die as a result of the MDS or its treatment [1, 2]. MDS therefore should not be considered as a preleukemic disorder, but as a separate neoplastic disease of the hematopoietic pluripotential stem cells that is characterized by a progressive clonal proliferation of abnormal precursors that demonstrate both impaired maturation and ineffective proliferation. The separation of MDS from AML, myeloproliferative neoplasms,

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aplastic anemia, and reactive disorders continues to be problematic, which is reflected in the evolving and at times inconsistent classification systems, prognostic models, and treatment options. A proposed revised 2016 WHO classification refines the morphologic interpretation and assessment of cytopenias addresses, and applies new diagnostic terminology for MDS. The WHO classification still relies mainly on the degree of dysplasia and the percent of blasts for disease classification and specific cytopenias now have only a minor impact on the MDS classification [3, 4]. Moreover the morphologic dysplasia may not correlate with the lineage specific cytopenias. Therefore the WHO has removed terms such a refractory anemia or refractory cytopenia and replaced it with the suffix Myelodysplastic Syndrome followed by the specific abnormality. The WHO also attempted to define and incorporate identified gene mutations associated with MDS and address the controversy and limitations of incorporating distinct mutations in the new proposed classification criteria. The WHO noted that that identification of dysplasia is subjective and may vary even among experienced hematopathologists [3]. Therefore when the dysplasia is subtle or limited to a single lineage it is important to consider other possible reactive and non-neoplastic causes of the dysplasia prior to making the diagnosis of MDS.

Pathogenesis and Etiology

MDS is one of the most common hematologic malignancies in western countries with an overall incidence of 3.5–12.6/100,000/year. The median age of MDS patients in western countries is 73 years, at the time of diagnosis, and the incidence increases with age. In individuals over the age of 70 years the incidence is between 15–50/100,000/year and is increasing with the aging population [5, 6]. However, the overall incidence of MDS is likely much higher due to difficulties in reporting, diagnosis, and classification [4]. The incidence of MDS is higher in men than women with the exception of the del (5q), which has a marked female predominance. In Asian countries, notably Japan and China, the median age of patients with MDS is between 40–50 years, some two decades earlier than in western countries [5, 7, 8]. The reason for the differences in the epidemiology of MDS in Japan and China is unclear but may, in part, reflect the variability and limitations of population-based databases. However, the role of environmental factors, industrial solvents and agricultural chemicals, and smoking may contribute to the observed 20-year differences in the epidemiology of MDS [9, 10].

Environmental agents have been implicated in the etiology of MDS. In case-controlled studies, there is an association between MDS and cigarette smoking, exposure to benzene, petroleum products, organic solvents, fertilizers, pesticides, and herbicides [10]. The associated between smoking and MDS may reflect that cigarette smoke contains benzene and other suspected carcinogens [9]. The MDS risk is related to the intensity and duration of smoking and may persist for up to 15 years after cessation of smoking [9, 10]. Ionizing radiation exposure is associated with a significant increase risk for the development of MDS. In the Nagasaki atomic bomb survivors risk for developing MDS was greater in individuals exposed at a younger age and occurred 40–60 years after exposure. In contrast to the reported radiation induced leukemia in Nagasaki which occurred 10–15 years after the exposure. The long latency may reflect the proposed multistep pathogenesis model for the development of MDS with age related changes and genetic instability associated with the prior radiation exposure [11]. In epidemiologic studies there was a linear radiation dose response for the development of MDS in Nagasaki atomic bomb survivors 40–60 years after exposure.

MDS is characterized by dysplastic, ineffective hematopoiesis. The bone marrow is typically hypercellular for the patient's age with peripheral cytopenias and an increase in hematopoietic precursors in the bone marrow and/or peripheral blood. The clonal origin of MDS has been confirmed by isozyme analysis of glucose 6 phosphate dehydrogenase (G6PD) in heterozygous females and more recently by molecular analysis of other loci such as the androgen receptor gene [8, 12]. Cytogenetic analysis has demonstrated recurrent genetic alterations that are prognostically important and next generation high throughput gene sequencing has defined a number of mutated genes in MDS [12] (Table 23.1). The appearance of clonal gene mutations arise

Table 23.1 Recurrent mutated genes in MDS

Mutated genes	Prognosis
Chromatin modification	
ASXL1	Unfavorable
EZH2	Unfavorable
UTX	Unfavorable
DNA methylation	
TET2	Neutral
DNMTA3A	Unfavorable
IDH1/	Unfavorable
RNA splicing	
SF3B1	Favorable
U2AF1/U2AF35	Unknown
SRSF2	Unfavorable
ZRSR2	Unfavorable
DNA repair	
p53(TP53)	Unfavorable
Transcription regulators	
RUNX1	Unfavoable
BCOR-L1	Unfavorable

Data from Ref. [23]

in hematopoietic stem cells and appear to be early events that are associated with clonal dominance. Specific gene mutations and the cytogenetic abnormalities can be demonstrated before the detections of morphological dysplasia or the clinical findings of MDS [13, 14]. The initial cytogenetic and somatic gene mutations are part of a multistep process that predisposes the pluripotential stem cell to secondary genetic events and the development of MDS [14]. Epigenetic alterations in one or more oncogenes including in the aberrant expression of specific tumor promotor, tumor suppressor, and transcription factor genes are associated with the progression of MDS [15, 16]. The diagnostic and prognostic role of specific gene mutations and aberrant methylation of epigenetic regulators, however, is still unclear. MDS progression is characterized by a progressive increase in chromosomal instability that leads to the development of aberrant clones and the emergence of complex karyotypes. Telomeres, noncoding repeated sequences at the ends of chromosomes, that function to stabilize chromosomes and prevent chromosomal breaks and aberrations critical in the maintaining normal hematopoiesis and are postulated to play a role in the progressive chromosomal instability in MDS [17]. Each somatic cell division is associated with loss of telomere length and the cumulative effects of telomere shortening leads to cell senescence. The shortening of telomeres is noted in patients with progressive, advanced MDS, with multiple complex karyotypic abnormalities. The genetic instability associated with shortening of telomeres may contribute, in part, to the leukemic transformation in some patients with MDS [17, 18]. Moreover, the alteration of telomere dynamics in hematopoietic stem cells may precede the clinical development of MDS [19]. However, the majority of patients that develop MDS are greater than 70 years old and loss of telomere length and function is part of the normal aging process [19].

The hematopoietic microenvironment may also play a role in the pathophysiology of MDS [20]. MDS is characterized by ineffective hematopoiesis and the increased susceptibility of hematopoietic progenitors to apoptosis. The bone marrow stroma responds to signals from the hematopoietic cells and is abnormal in some patients with MDS. Abnormalities of the bone marrow microenvironment and the hematopoietic stem cell niche may affect and promote apoptosis and telomere shortening in clonal hematopoietic cells [21]. The overexpression of TNF-a produced by MDS mononuclear cells can inhibit the growth of residual normal hematopoiesis and lead to increased cell death of normal precursors, and a growth advantage for the abnormal MDS precursors. The bone marrow in MDS patients has increased apoptotic cells which is most marked in the less proliferative, better prognosis, low risk subtypes of MDS.

Genetic, environmental, and exposure factors have been associated with an increased risk for the development of MDS [22]. Inherited constitutional genetic defects have been associated with up to 30% of children with MDS and related myeloproliferative disorders. Children with Shwachman–Diamond syndrome, Fanconi anemia, dyskeratosis congenitał, and neurofibromatosis type 1 have constitutional genetic defects that are associated with the increased risk for the development of both MDS and AML [25, 26].

Mutations of specific genes mediating DNA repair appear to predispose to the acquisition of secondary cytogenetic abnormalities that can lead to the development of MDS [23]. Somatic mutations occur in the majority of patients and may be associated with specific clinical features. Specific point mutations were associated with the clinical phenotype, specific cytopenias, disease progression, and overall survival. Genes encoding runt-related transcription factor 1 (RUNX1), tumor protein p53 (TP53), and neuroblastoma RAS viral oncogene homologue (NRAS) are associated with thrombocytopenia and an increased percent of bone marrow blast forms [23, 24]. Point mutations resulting in the activation of the specific genes (TP53, EZH2, ETV6, RUNX1, and ASXL1) are independent markers of poor prognosis and may, in part, explain the clinical heterogeneity of MDS. The TET family of genes maps to chromosome 4q24 and modulate hypomethylation by catalyzing an intermediate of DNA methylation that block the formation of silencing proteins to methylated DNA [13]. Mutations of the TET2 gene are found in a number of myeloid neoplasms including AML, MPNs, and MDS. In MDS TET2 mutation is the most frequent gene mutation occurring 20–30% of patients. Mutations of TET2 associated with loss of function may result in increased methylation and silencing of genes that are normally expressed. However, the prognostic impact of TET2 mutations on survival in MDS is unclear. The TET2 mutation is associated with a number of additional gene mutations and therefore may be one of the initial mutational events MDS. Recurrent mutations of epigenetic regulators, genes encoding the splicing machinery, spliceosomal components, and transcription factors are not unique to MDS and are found in a number of other myeloid neoplasms and occur across a spectrum of cytogenetic subgroups. It is controversial if these gene mutations are the primary events in MDS and are diagnostic and prognostic markers that are independent of other abnormalities [23, 24]. The frequency of the recurrent somatic mutations in MDS patients increases with progression of the disease and the subsequent development of secondary cytogenetic events and AML [25]. However, the etiological role of each of the somatic point mutations in the development of and progression of MDS is controversial and it is unclear if these mutations just reflect the genetic instability of the abnormal clone and its propensity to develop random genetic mutations [26, 27]. Moreover, similar somatic mutations have been noted in older normal individuals without evidence of MDS [27–29]. Somatic mutations resulting in clonal

hematopoietic cells are detected in greater than 10% of persons older than 70 years of age and the incidence of somatic mutations increases with age. These MDS associated acquired clonal mutations occur in hematopoietic cells of healthy older persons with normal blood counts and without evidence of dysplasia [29]. The presence of somatic mutations may confer an increased risk in the individual for the subsequent development of a hematological malignancy and are associated with an all cause-increased mortality [30–32]. The term "Clonal Hematopoiesis of Indeterminate Potential "(CHIP) is used describe these acquired clonal somatic mutations which are associated with hematologic malignancies in apparently healthy older individuals without any clinical features of a MDS or any myeloid malignancy. The natural history of individuals with CHIP is unclear and these persons should not be considered to have a malignancy. The revised 2016 WHO classifications addresses the controversy associated with somatic mutations and notes that the presence clonal somatic mutation alone, without other clinical manifestations of MDS, is not sufficient to make the diagnosis of MDS. While CHIP may represent a pre - malignant myeloid conditions, similar to monoclonal gammopathy of undetermined significance (MGUS) and multiple myeloma, the natural history of individuals with CHIP is not clearly defined. Testing for somatic mutations in healthy individuals should not part of routine clinical practice.

MDS patients have defects in a number of signal transduction pathways that appear to be related to the evolving ineffective hematopoiesis and epigenetic changes [33, 34]. These acquired abnormalities may contribute to the further dysregulation of progenitor cell cycle kinetics, response to cytokines, and the maintenance of DNA integrity, which results in progressive genetic instability. Abnormal regulation of microRNAs (miRNA) which function as epigenetic regulators of gene expression may play a role in the pathogenesis of MDS and alterations in miRNAs may be independent markers of prognosis [24].

MDS is associated with a number of immunoregulatory abnormalities including the development of autoantibodies and monoclonal gammopathies [35]. In subsets of patients with MDS, autoreactive T-cell clones are present that inhibit autologous erythroid and granulocytic colony growth. T-cell-mediated suppression of bone marrow growth and maturation is an important development of aplastic anemia and the hypoplastic variant of MDS [35, 36]. The incidence of MDS is increased in patients with autoimmune disorders and autoimmune disorders are more common in patients with MDS. The presence of autoimmune disorders is associated with a better overall survival and less frequent transformation to AML, The immunoregulatory abnormalities may also explain the response to immunosuppressive therapy in selected patients with MDS.

There are defined genetic predisposing factors in some MDS patients that relate to naturally occurring complex DNA polymorphisms in genes that mediate DNA repair and the metabolism of environmental carcinogens [37, 38]. In selected genetically predisposed individuals, MDS may arise as a result of cumulative environmental exposures and studies have linked the development of MDS and the nonfunction 609 C.T polymorphic allele of the NAD(P)Quinone oxidoreductase (NQO1) gene [40, 41]. These genes appear to play a critical role in detoxifying benzene and its metabolites. This association is controversial, but may explain the increased incidence of MDS in some patients exposed to organic solvents and benzene-containing compounds [39]. Similar controversial, but provocative results have been reported in the glutathione S-transferase (GST) genes that mediate the metabolism of cytotoxic and genotoxic agents [40].

A prior exposure to chemotherapy, especially alkylating agents and purine analogues is associated with an increased risk of MDS and AML (Table 23.2). The WHO identifies therapy-related MDS (t-MDS) as a separate category. Therapy related MDS represents approximately 10–20% of MDSs and MDS/MPNs [41]. The risk is, in part, related to the dose and duration of the cytotoxic therapy and generally occurs 3–7 years after the exposure. Patients who received combination radiation therapy and chemotherapy are at greater risk for the development of t-MDS [42]. Total body irradiation, administered as part of the preparative regimen for an autologous stem cell transplantation, is associated with an increased risk for MDS, and the combination of

Table 23.2 Cytotoxic drugs implicated in the development of MDS

Class/drug
Alkylating agents
Busulfan
Carboplatin
Cisplatin
Carmustine, Semustine, Lomustine
Chlorambucil
Cyclophosphamide
Dacarbazine
Mechlorethamine
Melphalan
Mitomycin C
Procarbazine
Thiotepa
Nucleoside analogs
Fludarabine
2-Chlorodexoyadenosine (Cladribine)
Antimetabolites
6-Mercaptopurine
Methotrexate
Azathioprine

Usually involving large fields, e.g., total body irradiation

Table 23.3 Cytogenetics in IPSS-R

Abnormality				
Prognostic subgroup/% of patients	Single	AML evolution/y	Median OS/months	Score
Very good (4%)	Del (11q)	NR	64	0
	−y			
Good (72%)	Normal	9.4	56	
	Del(5q)			
	Del(12p)			2
	Del(20q)			
Intermediate (13%)	Del (7q),+8,+19	2.5	31	
	i(17q)			4
	Any other			
	Independent clones			
Poor (4%)	Inv(3/t(3q)/de(3q)	1.7	18	6
	−7, double including			
	7/del7(7q), complex:3 abnormalities			
Very poor (7%)	Complex > 3 Abnormalities	0.7	8.4	8

OS overall survival, *NR* not reached
Data from Greenberg {L, Tuechler H, Sanz G, et al. Revised International Prognostic Scoring System for Myelodysplastic Syndromes. Blood 2012;120 (12): 2453–2465}

high-dose alkylator therapy and total body irradiation was associated with a 10–15% risk of t-MDS and secondary AML [41]. The MDS that occurs after chemotherapy, has a very poor prognosis [42]. Therapy-related MDS, is associated with deletions of chromosomes 5 and/or 7 and complex karyotypes. In contrast to the cytogenetic findings in AML, balanced cytogenetic abnormalities including translocations and inversions are rare in MDS. The cytogenetic abnormalities in MDS are important independent prognostic risk factors for overall survival and risk of the development of AML (Table 23.3). However, in the majority of patients with MDS there is no history of exposure to known mutagens, cytotoxic agents, or environmental agents and therefore the etiology of the syndrome remains idiopathic or unknown.

Diagnosis and Classification

The diagnosis and classification of MDS, similar to other myeloid malignancies, is evolving and incorporates new cytogenetic and molecular findings. The updated 2016 WHO classification attempts to addresses the heterogeneity of MDS and separates MDS from reactive processes and other malignant stem cell disorders. The revised classification refines the morphologic interpretations and addresses the influence of new genetic information in MDS diagnosis, classification and prognosis. The FAB (French–American–British) group was the first to define morphological criteria in the blood and bone marrow for the diagnosis and classification of MDS and was based only on morphology, and the percentage of blast forms in the blood and bone marrow. This classification system, although generally adopted at the time, was clinically and biologically inconsistent [43]. The separation of MDS from AML and other clonal disorders was based on an arbitrary number of blast forms. Moreover, many patients with MDS had clinical and laboratory features of AML, aplastic anemia, and myeloproliferative neoplasms which were not addressed in the FAB classification [44]. The FAB criteria also did not address the clinically important cytogenetic changes in MDS and were too variable to accurately predict prognosis, survival, or transformation to AML. The FAB classification remained as a widely accepted classification system for diagnosis of MDS for two decades. The FAB group defined five categories, of MDS based on morphologic dysplasia, cytochemical stains for iron to detect ring sideroblasts, and the percent of blast forms in the bone marrow and peripheral blood and included refractory anemia (RA), or refractory cytopenia, refractory anemia with ringed sideroblasts (RARS), refractory anemia with excess blasts (RAEB), chronic myelomonocytic leukemia (CMML), and refractory anemia with excess blasts in transformation (RAEB-T). The World Health Organization (WHO), in collaboration with the Society for Hematopathology and the European Association of Hematopathology in 2001, proposed a revision of the FAB morphological approach to the classification of MDS [45]. The WHO classification was updated in 2008 and most recently in 2016 [3, 46] (Table 23.4). The revised WHO classification attempts to combine clinical, morphologic, immunophenotypic, genetic and molecular features to define clinically and prognostically important subtypes. The current WHO classification is generally accepted and is incorporated in prognostic and treatment models of MDS.

Table 23.4 WHO 2008 and WHO 2016 classification of myelodysplastic syndromes

WHO 2008	Peripheral blood key features	WHO 2016 bone marrow key features
Refractory cytopenia with With unilineage dysplasia Dysplasia (RCUD) RA anemia (RA) Refractory neutropenia (RN) Refractory thrombocytopenia (RT)	<1% blasts	MDS with single lineage dysplasia (MDS-SLD)
Refractory anemia with ring sideroblasts (RARS)	Anemia no blasts	MDS with single lineage dysplasia and ring sideroblasts(MDS-RSSLD)
Refractory cytopenia with multilineage dysplasia (RCMD)	Cytopenia(s) <1% blasts No Auer rods	MDS with multilineage dysplasia and ring sideroblast(MDS-RSMLD)
Refractory anemia with excess blasts type 1 RAEB-1(RAEB1)	Cytopenia(s) <5% blasts No Auer rods	MDS with excess blasts-1(MDS-EB1)
Refractory anemia with excess blasts type 2 RAEB-2 (RAEB2)	Cytopenia(s) 5–19% blasts ± Auer rods	MDS with excess blasts-2(MDS-EB2)
MDS associated with isolated del(5q)	Anemia normal or high platelet count	MDS with isolated del(5q)
MDS, unclassifiable MDS-U	Cytopenias ≤ 1% blasts If no dysplasia, MDS-associated karyotype	MDS-U

The WHO classification included requirements for the type of specimens to be obtained, the assessment of blasts, assessment of blast lineage, and cytogenetic or mutational studies. The assessment of blasts in the peripheral blood (PB) and bone marrow (BM) should be obtained prior to any definitive therapy. Cytogenetic analysis and flow cytometry should be obtained, with additional material saved for later molecular genetic studies as needed [12]. The WHO lowered the threshold for percent of blasts to diagnose AML from 30% (FAB) to 20%. The percent of blasts should be derived from a 200-cell differential count of the peripheral blood smear and a 500-cell differential count of all nucleated bone marrow cells. The 2016 revised WHO classification changed the diagnostic criteria for myeloid neoplasms with erythroid dominance, defined as erythroid precursors ≥50% of all bone marrow cells. In the new classification the percent of blasts is based not on non-erythroid nucleated cells but on all nucleated bone marrow cells. This new criteria will result in cases previously diagnosed as the erythroid/myeloid subtype of acute erythroid leukemia to now being classified as MDS with excess blasts.

The FAB and the subsequent WHO classifications defined MDS as a clonal stem cell disorder characterized by ineffective, dysplastic hematopoiesis, with dysplasia in one or more hematopoietic cell lines and the dysplasia should be noted in >10% of cells in either the bone marrow or the blood. The WHO noted that morphological dysplasia is not specific or diagnostic of MDS and noted the difficulty in separating MDS from other disorders associated with cytopenias and dysplasia. The 2016 WHO classification recognized that dysplasia in excess of 10% may occur in some normal individuals and in other non-malignant hematologic disorders. The hematopathologist's identification of dysplasia is also variable and not always reproducible even by expert panels [47]. The WHO classifications like the original FAB classification is based on the degree of dysplasia and percent of blast forms in the blood and bone marrow. The WHO classification noted that the cell line demonstrating the most prominent dysplasia may not correlate with the cytopenia of the most affected lineage. The 2016 WHO classification therefore changed the descriptive terms such as refractory anemia and applied the new terminology Myelodysplastic syndrome followed by the "appropriate modifiers"; single or multilineage dysplasia, ring sideroblast, excess blast or the del(5q) cytogenetic abnormality (Table 23.4). There are, however, some of he morphological abnormalities that are more characteristic of MDS and are useful in confirming the diagnosis. The neutrophil and megakaryocytic dysplastic changes are the most specific and characteristic of MDS [3, 46, 48]. In the myeloid/neutrophilic dysmyelopoiesis the presence in the peripheral blood of the acquired, pseudo, Pelger–Huët anomaly is a frequent and useful characteristic finding in the MDS. This acquired abnormality resembles the inherited Pelger–Huët anomaly, therefore the designation of pseudo, and is characterized by mature neutrophils that are hypolobated with a single lobe or two joined by a thin band of chromatin [49]. Abnormal granulopoiesis is usually evident on the peripheral smear and includes hypersegmented neutrophils, with decreased or absent cytoplasmic granules The presence of dysmegakaryopoiesis including the presence of micromegakaryocytes in the bone marrow is also very suggestive of MDS rather than a reactive process [3, 45]. The finding of micromegakaryocytes and megakaryocytes that are the size of a myeloblast with one or two abnormal small nuclei in the bone marrow is one of the most characteristic, diagnostic and recognizable morphological features in MDS.

A majority of patients with MDS are asymptomatic at presentation and are usually diagnosed when they present with an unexplained macrocytic anemia (MCV > 102) with a absolute low reticulocyte count. Anemia is the most common presenting abnormality and patients may complain of the insidious onset fatigue and progressive dyspnea on exertion. Bone pain and weight loss are uncommon. While patients may be neutropenic and have dysplastic and impaired neutrophil function, infections are unusual at presentation. The physical examination is notable for the lack of adenopathy, cutaneous lesions, prominent splenomegaly, or hepatomegaly. The diagnosis of MDS relies largely on the morphological findings in the peripheral blood and the bone marrow (Table 23.5). MDS must be differentiated from other disorders that present with abnormalities of one or more cell line including aplastic anemia, myeloproliferative neoplasms, nutritional deficiencies, and autoimmune disorders (Table 23.6). AML in elderly patients may also present with progressive pancytopenia with rare circulating blast forms [56]. The differentiation of AML with dysplasia from MDS can be difficult. Patients with acute erythroleukemia may have prominent dysplastic erythroid precursors and may initially be diagnosed with one of the MDSs. Patients with hypoplastic AML can also be confused with one of the MDS subtypes. In prospective trials of MDS up to 15% of the patients with hypoplastic MDS were later reclassified as having AML [48, 50].

The finding of a clonal cytogenetic abnormality characteristic of MDS is important in establishing the diagnosis, assessing prognosis and differentiating it from other disorders [3]. The WHO defined specific cytogenetic abnormalities, even in the absence of diagnostic morphologic dysplasia, that where sufficient to make a diagnosis of MDS (Table 23.6). In these cases the cytogenetic abnormality must be demonstrated by conventional karyotyping and not by in fluorescence in situ hybridization (FISH) or sequencing technologies. FISH analysis has facilitated an accurate cytogenetic diagnostics and compliments karyotyping. FISH can be performed on the non-dividing cells and therefore can be performed on PB cells. FISH studies may be helpful in identifying specific rearrangements not recognized by banding studies alone. FISH also provides a convenient and sensitive method for monitoring patients with a specific cytogenetic abnormality. The WHO also defined some common MDS associated cytogenetic abnormalities as not sufficiently specific to diagnosis MDS in the absence of diagnostic morphologic findings. The presence of cytogenetic abnormalities +8, −Y, and del (20q) while frequently observed in patients with MDS was noted not to be diagnostic of MDS. In the updated 2016 WHO classification del(5q) remains the only cytogenetic or molecular genetic abnormality that defines a specific MDS subtype.

The patient's clinical findings and history are helpful in guiding the pathological evaluation. An analysis of the bone marrow is essential for the diagnosis of MDS. The bone marrow is typically hypercellular for the patient's age, confirming the ineffective hematopoiesis, and shows dysplastic features in one or several cell lines. A number of other disorders can present with finding similar to MDS (Table 23.7). A history of the patient receiving chemotherapy and or radiotherapy is important. Questions about environmental and occupational exposures should be noted, including the

Table 23.5 Morphologic features of dysplasia

Lineage	Peripheral blood	Bone marrow
Erythroid	Macrocytosis	Megaloblastic changes
	Elliptocytes	Nuclear budding
	Acanthocytes	Ringed sideroblasts
	Stomatocytes	Nuclear fragments
	Teardrops	Cytoplasmic vacuolization
	Basophilic stippling	Multinucleation
	Acquired thalassemia	
Myeloid	Pseudo-Pelger-Huet anomaly	Abnormal maturation
	Auer rods Blasts	Increase in monocytoid forms
	Hypogranulation	Abnormal localization of immature precursor (AILP)
	Hypersegmentation	
	Ring shaped nuclei-circle	Hypogranulation
	Cell blasts forms	Increased blasts
Megakaryocyte	Giant platelets,Megakaryocte fragments	Micromegakaryocyte
	Hypogranular or agranular	Hypogranulation
	Platelets	Multiple small nuclei
	Thrombocytopenia	Nuclear hypolobation
	Thrombocytosis	

Table 23.6 Recurring chromosomal abnormalities considered presumptive evidence of MDS in the absence of definitive morphological features

Abnormality	WHO-estimated frequency in MDS (%)
Unbalanced	
−7 or del(7q)	10
−5 or del(5q)	10
i(17q)or t(17p)	3–5
−13 or del(13q)	3
Del(11q)	3
Del(12q) or t(12q)	3
Del(9q)	1–2
idic(x)(q13)	1–2
Balanced	
t(11;16)	3 in t-MDS
t(3;21)	2 in t-MDS
t(1;3)	<1
t(2;11)(p21;q23)	<1
Inv(3)	
t(6;9)(p23;q34)	<1

Comments: Although +8, del(20q), and –y are common chromosomal abnormalities. In MDS, the presence of one of these three abnormalities as the sole cytogenetic abnormality in cases where morphological criteria for MDS are not met is not considered to be enough for presumptive diagnosis. t-MDS-Therapy related MDS [3]

Table 23.7 Differential diagnosis of myelodysplastic syndromes

Disorder	Comments
Congenital dyserythropoietic anemia	Inherited disorder characterized by marked dyspoiesis of mature and immature erythroid elements. Granulocytes and megakaryocytes unremarkable. Unstable hemoglobins normal karyotype.
Aplastic anemia	Both congenital (Fanconi) and acquired aplastic must be distinguished from hypocellular myelodysplasia. Karyotype normal in acquired aplastic anemia, rare cases with clonal abnormalities may actually represent hypocellular myelodysplasia. Severe malnutrition.
Hypocellular neoplasms	Hypocellular MDS must be distinguished from hypocellular AML. MDS with fibrosis may resemble primary myelofibrosis.
Megaloblastic anemia	Dyspoiesis restricted to megaloblastic changes. Normal karyotype. Use of antimetabolites need to confirm normal B_{12} level. Antimetabolites, chemotherapy.
Toxic exposure	Arsenic poisoning, alcohol, chemotherapy bone marrow cellularity normal; blasts are not increased. Normal karyotype; normal vitamin B_{12} and folate levels. Neurologic and gastrointestinal manifestations may dominate clinical picture.
Disorders with ringed sideroblasts	Chemotherapy, copper deficiency, hereditary sideroblastic anemia. Pyridoxine deficiency, zinc toxicity, alcohol toxicity, and in patients receiving Antituberculosis agents, chloramphenicol. Heavy metals.
Fibrotic disorders	Acute megakaryoblastic leukemia. Metastatic carcinoma with marked fibrosis, hairy cell leukemia, and primary myelofibrosis.
Viruses	Single, double or trilineage dyspoiesis common. HIV-1. Parvovirus. Karyotype normal
Autoimmune disorders	Myelodysplasia-like picture with dyspoiesis may be found in patients with underlying immune defects. May precede evolution to red cell aplasia. SLE, rheumatoid arthritis.
Paraneoplastic syndromes	Myelodysplasia-like picture (usually resembling chronic myelomonocytic leukemia). Occasionally noted at diagnosis in patients with solid tumors (lung, colon, prostate, and gastric carcinoma and lymphomas). Distinct from therapy-induced bone marrow neoplasms in carcinoma patients.
Bone marrow regeneration	Abnormal localization of immature precursors, dyspoiesis and increased immature myeloid cells may be transient phenomena after aggressive therapy. Increased blasts from recovering bone marrow after cytoxic chemotherapy, drug toxicity or bone marrow transplantation. Karyotype normal.
Colony-stimulation factor therapy	Transient increase in blasts in blood and bone marrow. Clusters of blasts on core biopsy. Hypolobated neutrophils and toxic granule in neutrophils may be seen.
Drugs	After purine analog therapy, Zidovudine and other antivirals Dilantin, methotrexate, valproic acid, sulfasalazine, mycophenolate, etc.

patient's smoking history. An occupation history is important because excess cases of MDS have been reported in agriculcultural and industrial workers.

Clonal cytogenetic abnormalities are found in 38–78% of patients with de novo MDS and greater than 80% of t-MDS patients [50]. Cytogenetics are critical in evaluating disease progression and prognosis. All of the currently used prognostic scoring system acknowledge the importance of specific cytogenetics abnormalities to define prognosis and plan therapy. In addition, the acquisition of new cytogenetic abnormalities is generally associated with progression of the disease and a poor prognosis. Although anemia is the most common presenting laboratory feature in patients with MDS, approximately 50% of patients will demonstrate an abnormality of more than one cell line [51]. The WHO thresholds

for defining cytopenias remain the same in the new 2016 classification: hemoglobin <10 g/dL, platelets <100 × 10^9/L and a absolute neutrophil count of <1.8 × 10^9/L. Patients may be at risk for bacterial infection generally due to qualitative abnormalities of neutrophil function and platelet functional abnormalities are associated with increased risk for bleeding even with an adequate platelet count. Iron studies may demonstrate increased iron stores even in patients who have not received red cell transfusions. The use of flow cytometry to determine the percent of blasts, assessment of CD34+ cells, is not recommended by WHO criteria as a substitute for the visual inspection of the bone marrow, unless the aspirate was of poor quality. The percent of CD34+ cells generally correlates with morphologic examination of routine bone marrow aspirate and peripheral blood smear; however, the WHO noted that not all leukemic blasts express CD34 and hemodilution and processing artifacts can yield misleading results. Multiparameter flow cytometry was recommended to determine the blast lineage and to determine aberrant antigen expression. However, it remains unclear if these changes are specific for the diagnosis of MDS as they occur in other myeloid neoplasms [51].

Immunophenotyping combined with cytogenetics and morphology help in the diagnosis and assessing the possible evolution of MDS to AML [52]. An increase in the percentage of CD34+ cells or CD117+ positive cells may help in documenting the progression to AML in a low-grade MDSs. The WHO, however, while noting that aberrant antigen expression patterns are common in MDS did not consider the phenotypic abnormalities sufficient, in the absence of conclusive morphologic and/or cytogenetic abnormalities, for the diagnosis of MDS. The finding of three or more phenotypic abnormalities involving one or more of the myeloid lineages should be considering suggestive, but not diagnostic of MDS. Patients whose cells demonstrate aberrant immunophenotypic markers should be followed for morphologic features sufficient to diagnose MDS. The use of additional studies including FISH analysis and gene mutational analysis should be performed as clinically indicated. The myelodysplastic/myeloproliferative neoplasm (MDS/MPN) group is an overlap disorder reflecting that some patients have features of both MDS and MPN and patients present with a clinical picture that demonstrates both increased proliferation and dysplastic and ineffective maturation. The classification of MDS/MPN reflects difficulty-separating MDS from other myeloid neoplasms.

Patients with MDS/MPN usually present with a leukocytosis and hepatosplenomegaly. The MDS/MPN category includes four defined entities: chronic myelomonocytic leukemia (CMML), atypical chronic myelogenous leukemia (a CML-Philadelphia chromosome negative), juvenile myelomonocytic leukemia (JMML), and a more heterogenous group of unclassifiable MDS/MPN (U-MDS/MPN). The criteria for MDS/MPN with ring sideroblasts and thrombocytosis, MDS/MPN-RS-T, (previously known as RARS-T) is better defined and includes thrombocytosis, ≥450 × 10^9/L associated with anemia, erythroid dysplasia, with ring sideroblasts in ≥15% of erythroid precursors. Megakaryocytes with features of a chronic myeloproliferative neoplasm that are hyperlobulated and atypical and are increased in number with large or giant forms with lobated staghorn like nuclei and occur in lose clusters or found adjacent to the endosteum or within sinusoids and important pathologic finding [52]. The spliceosome gene mutation SF3B1, associated with ring sideroblast, is frequently present in cases of MDS/MPN-RS-T but the subtype still requires ≥15% ring sideroblasts. The JAK 2 V617F mutation has been noted in some of the cases of MPN/MDS but the proliferative potential of most cases appear to be related to aberration in the RAS/MAPK or other signaling pathways and the JAK 2 mutation is not the primary oncogenic event to explain the MPN [42, 66]. The prognosis of patients with MDS/MPN-RS-T is better than most of the MDS/MPN disorders. The transformation to AML is uncommon and unlike the MPN there does not appear to be an increase risk of thrombotic or bleeding complications associated with the elevated platelet count. The treatment options for this subtype are not clear and additional studies are needed to further define the clinical course. In addition to the JAK 2 mutation some patients demonstrate the MPL mutation and both mutations appear to correlate with the elevated platelet count. In general the MDS/MPN disorders have a variable clinical course but are generally associated with poorer prognosis than their myeloproliferative neoplastic counterpart and an increased incidence of leukemic transformation. In MDS/MPNs the karyotype is usually normal or demonstrates a typical MDS cytogenetic abnormality. Gene mutations are common in the MDS/MPNs and can be helpful in assessing difficult cases with a normal karyotype. The 2016 WHO classification, however, noted that gene mutations even of the most commonly mutated genes, SRSF2, TET2, ETV6 and ASXL1 is not sufficient proof of a neoplastic disorder because gene mutations occurs in healthy older persons with undetermined significance. While some of these mutations are prognostically important and define aggressive disease, and have been incorporated into new prognostic scoring systems they should not be the sole determinant of a neoplastic disorder [53].

The diagnosis of myelodysplastic/myeloproliferative neoplasm unclassifiable (MDS/MPN-U) includes patients with features of both myelodysplasia and myeloproliferation who cannot be assigned to a more specific category. Patients must meet the criteria of one of the categories of MDS and demonstrate prominent myeloproliferative features without a prior MPN or MDS. The WHO emphasizes that the diagnosis of MDS/MPN-U should not be made in patients who have recently recovered from cytotoxic chemotherapy or have received recent growth factor therapy.

A follow-up evaluation in these patients is essential to demonstrate that the changes in the PB and BM are independent of recent treatments or recovery from chemotherapy. The features that distinguish this subgroup include an elevated platelet count or WBC without evidence of BCR–ABL1 or rearrangement of PDGFRA, PDGFRB or FGFR1, del 5q, t (3; 3), or inv3. In most patients, the karyotype is normal and no abnormal mutational studies are noted but the JAK2 V617F mutation may be present [54].

MDS with Fibrosis and Hypocellular MDS

The WHO classifications did not define a number of MDS subtypes that are generally recognized as clinically distinct. The categories MDS with marrow fibrosis and hypocellularity MDS are distinct disorders but the WHO noted that because they lack a consensus on the precise definition or the importance of these findings as distinct entities they on not included in the current classification. A subset of patients with MDS present with a hypocellular bone marrow (less than 15% cellularity on bone marrow biopsy) and minimal dysplasia. Hypocellular MDS must be differentiated from aplastic anemia and hypocellular AML [55]. Hypocellular MDS and aplastic anemia may also be pathophysiologically related [54]. The similarity of both disorders is suggested by their response to immunosuppressive therapy. Hypocellular MDS may represent an intermediate stage in the evolution of aplastic anemia. However, hypocellular MDS in contrast to aplastic anemia tends to occur in older patients, with a more gradual onset and dysplasia involving more than one cell line. In contrast to aplastic anemia dysplastic megakaryocytes are more prominent in hypocellular MDS. Megaloblastic red cell precursors, pancytopenia, and the presence of a paroxysmal nocturnal hemoglobinuria clone (PNH)—occur in both hypoplastic MDS and aplastic anemia and are therefore not helpful diagnostic features A characteristic MDS cytogenetic abnormality sometimes helps to define hypocellular MDS but similar findings may also be present in aplastic anemia [48]. Patients with a PNH clone and hypocellular MDS respond better to immunosuppression.

MDS with fibrosis (MDS-F) can be difficult to differentiate from primary myelofibrosis (PMF), acute megakaryocytic leukemia or acute panmyelosis [55]. The bone marrow is usually not aspiratable (dry tap) and the morphological findings of dysplasia may be difficult to identify on the bone marrow biopsy. Splenomegaly and leukoerythroblastosis on peripheral smear are unusual in MDS-F and when present suggest the diagnosis of PMF. Staining the marrow biopsy and circulating blast forms with megakaryocytic lineage-specific antigens can identify abnormal megakaryoblasts [55]. The finding of diffuse fibrosis in MDS is associated with a poor prognosis and greater than 2+-reticulum fibrosis, as defined by the European consensus system, is an independent negative prognostic marker [52]. The diagnostic challenges that such cases present are discussed by the WHO with a recommendation that hematopathologists should specifically comment upon the hypocellularity or extensive fibrosis in interpretative reports. The WHO classification, however, does not recognize hypocellular MDS and MDS-F as distinct entities as the sub-classification of these cases can be problematic and there is no accepted agreement on the diagnostic features associated with these two disorders. Immunohistochemical stains for CD34 on the biopsy may demonstrate excess blasts and may help in identifying these patients. Bone marrow fibrosis is associated with a higher red cell transfusion requirement, multilineage dysplasia, pancytopenia, and a poor prognosis. The 2008 and 2016 WHO classification attempts to address these issues, about when to call a disorder a separate diagnostic entity, and raises the question of when sufficient clinical characteristics or morphologic findings are distinctive enough to warrant a separate diagnostic category.

The recognition and enumeration of blast cells is of critical importance for the diagnosis of AML, MDS and defining the subtypes of MDS and requires a differential blast count on 500 cells on the aspirate to be 20% or more for a diagnosis of AML (either de-novo or evolved from a prior MDS). If a concomitant non-myeloid neoplasm (i.e., plasma cells) is present those cells should be excluded from the count used to evaluate the percent of blast forms. If an aspirate is not available a touch preparation of the biopsy may yield valuable cytologic information, but differential counts from touch preparations may not be representative and should be confirmed on the bone marrow biopsy. The definition of a blast cell can be difficult in MDS. The WHO did not specifically define the definition of a blast, but noted that blasts can be granular or agranular [54]. Myeloblasts are defined by a high nuclear/cytoplasmic ratio, visible nucleoli and usually fine nuclear chromatin. Nuclear shape can be a variable with basophilic granule, or Auer rods, aggregates of lysozymes, may be noted but no Golgi zone is detected. Granular blast cells must be distinguished from promyelocytes and the principal distinguishing characteristic of the normal promyelocyte is the presence of a visible Golgi zone [54]. Dysplastic promyelocytes have the recognizable features of promyelocytes including round, oval or an indented nucleus that is often eccentric with decreased granules or irregular distributed granules and a poorly developed Golgi zone. Determining the overall percentage of blasts in the context of marked erythroid hyperplasia can be problematic. In the updated 2016 WHO classification the percent of blasts is based on all nucleated cells not just the non-erythroid cells. This new criteria will results in most cases previously diagnosed as the erythroid/myeloid subtype of acute erythroid leukemia in the 2008 WHO criteria now being classified as MDS with excess

blasts. In the updated 2016 WHO classification the cytogenetic abnormalities that are MDS defining remain unchanged. While acknowledging the prognostic importance of genetic abnormalities in MDS, in the 2016 WHO criteria only the del(5q) cytogenetic or molecular genetic abnormality defines a specific MDS subtype even if an there is an additional cytogenetic abnormality [3].

The 2016 WHO criteria noted the association between ring sideroblasts and an SF3B1 mutation. The SF3B1 mutation appears to be a early initiating event in the development of MDS and manifests a distinct gene expression profile and correlates with a favorable and indolent course [56]. Patients with MDS carrying the SF3B1 mutation have a more homogeneous phenotype characterized by isolated erythroid dysplasia and the presence of ring sideroblasts. Moreover, the actual percent of cells with ring sideroblast does not affect the prognosis.

The 2008 WHO classification redefined a number of new subgroups [57, 58]. Three categories of refractory cytopenia with unilineage dysplasia (RCUD) are defined: Refractory anemia (RA), refractory neutropenia (RN) and refractory thrombocytopenia (RT). These subgroups do not have an increase in blasts and involve a single lineage. In the 2016 WHO classification these subgroups are part of MDS with Single lineage Dysplasia (MDS-SLD). Other causes of dysplasia need to be addressed and excluded before the diagnosis of single lineage MDS is established (Table 23.7). The expansion of the erythroid component can occur in non-neoplastic disorders, including hemolytic anemia, iron deficiency, and B12 or folate deficiency. Erythropoietin administration may also lead to a marked expansion of the erythroid precursors. Simultaneous or chronologically close administration of erythropoietin and granulocyte growth factors may lead to erythroid hyperplasia with an increase in pronormoblasts and myeloblasts. Therefore, the pathologist must have knowledge of the patient's clinical history, including the administration of recent chemotherapy or growth factors when evaluating the bone marrow. Other causes of erythroid dysplasia include alcohol abuse (vacuolated erythroid precursors and ring sideroblasts), anti-tuberculosis medications and chloramphenicol. Isolated unilineage dyspoiesis should raise suspicion for secondary causes, including disorders not associated with prescription medications as seen in zinc over consumption leading to copper deficiency. Zinc competes with copper for its carrier, ceruloplasmin. Primary copper deficiency can present with pancytopenia with marked vacuolation of erythroid and myeloid precursors, megaloblastoid changes in red cell precursors and ring sideroblasts [59, 60] (Table 23.6). The 2016 WHO classification notes that the prognostic importance of genetic finding in MDS and the expanding knowledge of the clinical importance of recurring mutations in MDS but notes that the finding of one or more somatic mutation is not considered diagnostic of MDS even in a patient with unexplained cytopenias where these mutation may be frequently found [3].

In patients with MDS who lack the appropriate finding of any defined MDS category diagnosed with subtype, MDS, unclassifiable (MDS-U). MDS-U has no specific morphological features but includes MDS with the presence of 1% blast forms in the peripheral blood and <5% blasts in the bone marrow. An occasional blast can be found in healthy individuals and to avoid over interpreting the rare blast form the WHO notes that blasts must be noted in the peripheral blood on two or more successive evaluations to confirm the diagnosis of MDS-U. In addition the diagnosis of MDS - U can be made in cases with 1% or fewer blasts in in the blood and <5% blasts in the bone marrow with unequivocal dysplasia in <10% of one or more myeloid lineages but have a diagnostic cytogenetic abnormality. MDS-U is a very heterogenous subtype and patients have a variable clinical course and should be reclassified if they develop findings characteristic of a specific MDS subtype. The MDS-U subtype may represent the early phase of one or more specific MDS subtypes but with nonspecific morphological features of MDS.

Idiopathic Cytopenia(s) of Undetermined Significance and Idiopathic Dysplasia of Uncertain Significance

Patients who present with cytopenias but lack the diagnostic criteria of MDS, but in whom the diagnosis of MDS is suspected, are classified with the diagnosis "Idiopathic Cytopenia(s) of Undetermined Significance" (ICUS) [61]. The criteria for this group of disorders reflects the diagnostic uncertainty in these patients. A key distinction of ICUS from other potential precursor conditions such as MGUS, monoclonal B-cell lymphocytosis (MBL) and T cell clonality of undetermined is that an ICUS designation does not necessarily imply a clonal disorder. Limited data are available about the frequency or natural history of ICUS and reflects that some patients present with persistent cytopenia but lack the diagnostic features of MDS. The term idiopathic dysplasia of uncertain significance (IDUS) was proposed to describe a group of patients with dysplasia but no or only mild cytopenias [61]. In contrast to ICUS, patients with IDUS demonstrate dysplasia in >10% of cells in one or more lineage with or without a MDS-related karyotype but without persistent cytopenias. ICUS and IDUS have very variable courses and it is unclear if all the patients will ultimately develop a defined subtype of MDS or another myeloid neoplastic disorder. Moreover, it is unclear if these disorders are mutually exclusive or if the classification of a potentially premalignant disorders will provide meaningful prognostic or diagnostic information for patients in whom no cause for the cytopenia

Table 23.8 New terms for patients who do not meet the criteria for MDS

ICUS	*Idiopathic Cytopenias of Undetermined Significance*. The natural history of patients with ICUS is unclear. May have clonal or nonclonal hematopoiesis (clonal ICUS vs nc ICUS) as defined by somatic mutations or non- diagnostic cytogenetic findings by karyotype or FISH. A proportion of patients with ICUS with develop MDS or another myeloid malignancy. Much more common than MDS and most patients will not develop a myeloid malignancy.
IDUS	*Idiopathic Dysplasia of Undetermined Significance*. Dysplasia without cytopenia. Need to confirm the dysplasia over a 3–6 month period. Unclear clinical course and prognosis and frequently associated with benign conditions.
CHIP	*Clonal Hematopoiesis of Indeterminant Potential*. Age dependent somatic mutations in persons without a known hematologic disorder. The prevalence of CHIP increases greatly with age over 10% in person over the age of 70 years. Most with never develop MDS but is associated with an increased incidence of myeloid malignancies and all-cause mortality. In most persons may be an incidental findings relating to aging.
CCUS	*Clonal Cytopenias of Underdetermined Significance*. Have one or more cell line decrease, hemoglobin <11 g/dL, absolute neutrophil count (ANC), 1.5×10^9/L, platelet count < 100×10^9/L. persons have an acquired chromosomal abnormality not diagnostic of a hematologic malignancy and/or the presence of a somatic mutation with a variable allele fraction ≥2% in hematologic malignancy—Associated gene in the peripheral blood or bone marrow. Most persons had somatic mutations fraction of >10% similar to lower risk MDS patients, including mutated genes associated with high risk MDS including TP53, ASXL1,RUNX1 and DNMT3A. More frequently diagnosed than MDS but less than ICUS. No evidence of dysplasia. The natural history for most persons in unknown unlikely that all will develop MDS or other myeloid malignancy.

is found. The proposed term Clonal Cytopenia of Undetermined Significance (CCUS) has been proposed for cases with somatic mutations, or a non-diagnostic chromosomal abnormality, defining a clonal population in the bone marrow, but without dysplasia and one or more cytopenia in the blood (Table 23.8) [62].

MDS Subtypes

MDS with Ring Sideroblasts (MDS-RS)

MDS with ring sideroblasts (MDS-RS) is characterized by anemia, erythroid dysplasia and >15% ring sideroblasts of bone marrow erythroid precursors. There is generally no or minimal dysplasia in the non-erythroid precursors. Myeloblasts comprise <5% of the nucleated BM cells and are not present in the PB. In the 2016 WHO criteria MDS-RS is associated with recurrent mutations of the spliceosome gene SF3B1. The classification of MDS-RS was changed to include MDS cases with ring sideroblasts and multilineage dysplasia. This change reflected the link between ring sideroblasts and an SF3B1 mutations. In the 2016 classification if the SF3B1 mutation is identified then the diagnosis of MDS—RS can be made even if the ring sideroblast comprise only 5% of nucleated erythroid cells. If the SF3B1 mutation is lacking then ≥15% ring sideroblasts of nucleated erythroid cells is still required. MDS-RS subtype is divided into two groups; a group with single lineage dysplasia (MDS-RS-SLD), previously classified as refractory anemia with ring sideroblasts, and second group with multilineage dysplasia (MDS-RS-MLD), previously classified as refractory cytopenia with multilineage dysplasia. Patients with MDS—RS who lack the SF3B1 mutation have a more heterogenous phenotype, a high prevalence of TP53 mutations and less favorable prognosis. The SF3B1 mutation is early event in the development of MDS and may be an important therapeutic target in MDS-RS [58]. MDS-RS constitutes approximately 10% of cases of MDS. A majority of patients present with a moderate normochromic or macrocytic anemia. The PB frequently reveals dimorphic red cells due to a small population of microcytic and hypochromic red cells. Basophilic stippling and Pappenheimer bodies may be noted in red cells. Dysplasia is present in <10% of neutrophils and platelets. The bone marrow is usually hypercellular for the patient's age and demonstrates erythroid hyperplasia. The iron stain, Prussian blue staining, reveals ring sideroblasts that surround at least a third of the nuclear circumference. Iron stores are generally increased even in the absence of red cell transfusions. The number of CD 34+ cells is normal and most patients do not demonstrate a cytogenetic abnormality. Ring sideroblasts may be seen in a number of other, non- MDS-related disorders including lead poisoning, drugs including isoniazid which inhibits delta aminolevulinic acid (ALA) dehydratase activity and block hemoglobin formation resulting in ring sideroblast formation [45]. A number of acquired and hereditary conditions are associated with ring sideroblast formation and should be excluded before a diagnosis of MDS-RS is established. In RARS the ring sideroblasts and increased iron stores reflect abnormal iron metabolism in the erythroid lineage resulting from the ineffective erythropoiesis. The overall prognosis for patients with MDS-RS is 69–108 months and less than 2% of cases transform into AML. Progressive anemia requiring transfusion support is frequent and in select patients iron chelation therapy should be considered early in the clinical course to prevent iron overload and end organ failure. However, the overall beneficial effects of early iron chelation has not been demonstrated to improve survival in prospective randomized studies and therefore remains controversial [63].

MDS with Single Lineage Dysplasia and MDS with Multilineage Dysplasia

This group of disorders was previously know as Refractory anemia and refractory anemia with multilineage dysplasia in the 2008 WHO classification and is now classified as MDS-SLD and MDS-MLD. The difference between these subtypes reflects either a single or 2 or more lineages demonstrate dysplasia. These groups constitute approximately 30% of MDS cases and are characterized by one or more cytopenias and dysplastic changes in one two or more of the myeloid lineages. Blasts are rare, <1%, in the PB and <5% in the BM. Auer rods are not present in either the PB or BM. The anemia is usually macrocytic or normocytic with prominent granulocytic dysplasia including hypo granularity, nuclear shape abnormalities including hypo-lobation, acquired pseudo Pelger-Huet anomaly, and abnormal nuclear clumping. The bone marrow is usually hypercellular for age of the patient with <5% blasts. Erythroid precursors may demonstrate cytoplasmic vacuoles and marked nuclear irregularity including internuclear bridging and nuclear budding. The BM may have variable number of ring sideroblasts but less than 15%. The previously described WHO category of refractory cytopenias with multilineage dysplasia with ring sideroblasts has been omitted and incorporated in MDS-RS-MLD. Megakaryocytic dysplasia includes hypolobated and non-lobated nuclei, multinucleated and micromegakaryocyte, megakaryocytes with non-lobated or bi-lobed nuclei. Clonal cytogenetic abnormalities are present in up to 50% of patients and are important in defining the prognosis. The prognosis is related to the degree of cytopenias and cytogenetic abnormalities.

MDS with Excess Blasts 1(MDS-EB1 and MDS-EB2)

MDS-EB1 and EB2 comprises 40% of cases of MDS and is divided into MDS-EB1 and MDS-EB2 on the basis of the number of blasts and the presence or absence of Auer rods. EB 1 is defined by 5–9% blasts in the BM or 2–4% blasts in the PB and no Auer rods and EB2 is defined by 10–19% blasts in the BM or 5–19% blasts in the PB. The presence of Auer rods confirms the diagnosis of EB2 irrespective of the percent of blast forms. Most patients present with symptoms of BM failure including anemia, bleeding or neutropenia. The PB generally shows dysplastic changes in all three-cell lines and is typically hypercellular for age of the patient. Erythroid precursors may be increased with megaloblastoid changes and ring sideroblasts. The excess blasts define these subtypes. Dysmegakaryopoiesis is a frequent finding including micromegakaryocytes and abnormal megakaryocytic clustering. Blasts may form abnormal aggregates or clusters that are located away from trabeculare and vascular structures, a histologic finding previously referred to an abnormal localization of immature precursors (ALIP). Immunohistochemical staining for CD34 may help in identifying blast forms. Clonal cytogenetic abnormalities are observed in 30–50% of cases including +8, -5, del(5q),−7, del(7q), del (20q) and complex karyotypes. Fibrosis may be present and results in a dry tap. The presence of fibrosis should be noted and the finding of extensive fibrosis is an independent negative prognostic marker in MDS [3]. MDS-EB1 and 2 frequently progress to AML, 25% and 33% respectively for MDS-EB1 and MDS-EB2 respectively. The median survival is approximately 16 months for MDS-EB1 and 9 months for MDS-EB2. The survival is dependent on the number of blast forms. Cases with >5% blasts and a complex karyotype have a median survival of ≤3 months similar to AML with myelodysplastic changes.

Myelodysplastic Syndrome with Isolated del (5q)

Heterozygous, interstitial deletions of the long arm of chromosome 5 (5q) are the most common cytogenetic abnormality in patients with MDS. Del 5q is associated with a consistent clinical phenotype previously known as the 5q- syndrome in a subset of patients. Abnormalities in chromosome 5 occur in approximately 25% of MDS patients, but the incidence of the originally described 5q- syndrome is much less frequent [63]. The 5q- syndrome was originally described in patients with a macrocytic anemia, dyserythropoiesis and erythroid hypoplasia in the bone marrow and a normal to elevated platelet count, hypolobated megakaryocytes and an intestinal deletion involving the long arm of chromosome 5. In addition the 5q- syndrome in characterized by the absence of circulating myeloblast and therapeutic sensitivity to treatment with lenalidomide. The deletion of 5q in MDS does not necessarily equate to the clinical 5q- syndrome. Both the original 5q- syndrome and del (5q) MDS respond to lenalidomide and the revised WHO changed its definitions to this cytogenetically defined subset from 5q- syndrome to MDS with abnormality del(5q) [64, 65]. The deletion occur on a single chromosome resulting in a heterozygous (haploinsufficient) with the unaffected chromosome 5 contains the normal allele of all the genes contained in deleted segment. None of the genes on the nondeleted chromosome 5q are mutated or undergo homozygous inactivation in MDS patients. The recurrent haploinsufficiency for critical genes within the common deleted regions (CDR) on chromosome 5q is the basis for the unique pathological phenotype that results in the MDS subtypes del (5q). Haploinsufficiency of the RPS 14 gene on the long arm of chromosome 5 (5q) leads to activation of the P53 pathways and the development of the characteristic macrocytic anemia. The CDR on chromosome 5 and the breakpoints and size of

the deletions in the original 5q- syndrome patients and the del (5q) patients with advanced MDS and AML are variable. The CDR of the 5q–syndrome the interstitial deletion occurs in a 1.5 MB region at 5q32–33. The region contains genes for the ribosomal protein RPS14 and three micro RNAs mrR-143, miR-145, and mir-146. The non-allelic deletion of the RPS14 gene encodes for a component of the 40 s ribosome and is critical for the development of the macrocytic anemia. In contrast, patients with del (5q), and additional chromosomal abnormalities and excess blast have a different clinical course and response to treatment [85]. Patients with a deletion of 5q- and AML have large interstitial deletions that overlap the CDR of the 5q-syndrome and low risk del (5q) However, in high risk MDS and AML the deleted region was in a more distal CDR in the 5q32–33 region. Lenalidomide selectively inhibits the Del (5q) clone and results in RPS 14 inactivation of the p53 pathway. Lenalidomide exerts unique karyotype specific activity in Del (5q) MDS but does not eradicate the Del (5q) stem cell population in all patients. The inactivation RPS14 leads to defective erythropoiesis and increased apoptosis in erythroid progenitors. Moreover, in the congenital disorder Diamond- Blackfan anemia the down regulation of an another ribosomal gene (RPS19) is critical in the development of the erythroid hypoplasia and chronic anemia [65]. The down regulation of RPS14 may not be the sole genetic event underlying the del 5q- syndrome and alteration of other genes in the commonly deleted segment in 5q- may be required. The tumor suppressor SPARC (Secreted Protein Acidic and Rich in Cysteine) gene is located in the del 5q31 region. SPARC has tumor suppressor, antiproliferative, and anti angiogenesis properties and may also be important in this syndrome [64]. The loss of additional genes that code for these and other factors appear to contribute to the development of this syndrome and its unique response to the immune modulatory drug, lenalidomide. MDS with isolated del 5q syndrome is frequently associated with morphological features of MDS-SLD. Thrombocytosis and anemia is occasionally seen and when present is suggestive of the del 5q chromosomal abnormality. The bone marrow aspirate and biopsy are typically hypercellular for the patient's age with erythroid hypoplasia and dysplastic erythropoiesis. Ring sideroblasts may be present but <15% of erythroblasts. A del(5q) subtype should be suspected in patients who present with a refractory macrocytic anemia, with a normal or mildly low leukocyte counts, and thrombocytosis (a platelet count $>400 \times 10^9$/L). In contrast to the other MDS subtypes, where the mononuclear megakaryocytes are smaller (micromegakaryocytes), in the MDS with del 5q the megakaryocytes are bilobed or non-lobulated but of normal size (mono lobulated). The MDS with del 5q has a marked female predominance (70%), and rarely transforms to AML. A majority of patients have progressive anemia and become red cell transfusion dependent and rarely respond to growth factors including erythropoietin. Lenalidomide is the treatment of choice and results in transfusion independence in over two thirds of cases with durable clinical and cytogenetics responses [42].

Clinical and Laboratory Features of MDS

Blood and Bone Marrow Findings

Red Cells/Anemia

Macrocytosis or a macrocytic anemia with a low reticulocyte count is common in MDS and reflects the ineffective erythropoiesis. Impaired red cell maturation has been associated with acquired abnormalities of globin chain synthesis, and red cell enzymes. PNH has been described in the setting of MDS, and these patients have many of the typical diagnostic features of PNH including a defect in the synthesis of the glycosylphosphatidylinositol (GPI)-linked surface protein, but lack the ongoing red cell hemolysis and thrombotic complications associated with PNH. Cases may have abnormalities in the size and shape of red cells including basophilic stippling (red cell inclusions composed of ribonucleoprotein and mitochondrial remnants), Pappenheimer bodies (basophilic iron-containing granules peripherally located in red cells), macro-ovalocytes, teardrop forms, and nucleated red cells. The bone marrow may reveal multinuclear fragments, inter-nuclear bridging, and nuclear cytoplasm asynchrony.

Neutrophils

Qualitative abnormalities of neutrophil function are a common feature of MDS and may explain the increased risk for bacterial infections. Morphological abnormalities include hypo-granular and hyposegmented neutrophils, which are associated with a negative peroxidase reaction and decreased myeloperoxidase activity. The neutrophils are hyposegmented and may be confused with band forms. Nuclear fragmentation and nuclear-cytoplasmic asynchrony in early myeloid precursors may be a prominent feature in the bone marrow. Dysplastic myeloid precursors can be difficult to distinguish from blast forms and therefore a pathologist experienced in the interpretation of MDS should review the bone marrow.

Platelets

Thrombocytopenia and abnormal platelet function occur in MDS. Thrombocytopenia is an adverse prognostic feature independent of other prognostic factors [66–68]. While thrombocytopenia is associated with poor performance status and other unfavorable prognostic variable bleeding complications are underreported. Thrombocytopenia ($<100 \times 10^9$/L) has been reported in 66% of patients and was associated with a 24% incidence of deaths from hemorrhage. Impaired platelet function may also explain the

increased risk of bleeding in patients with MDS. Spontaneous bruising and bleeding after surgery or mild trauma occurs in MDS patients with a normal or slightly depressed platelet counts. Dysplastic platelets and abnormal megakaryocytes are important diagnostic features and help in distinguishing MDS from other disorders. Giant platelets, and agranular (grey platelets) and megakaryocytic fragments in the peripheral blood film are important diagnostic features of MDS.

Bone Marrow Findings

A bone marrow aspirate and biopsy is essential for the making diagnosis of a MDS and to define the MDS subtype. Abnormal distribution of cells is often present; erythroid islands may be absent or very large. Granulocytic precursors may be clustered centrally rather than their normal paratrabecular distribution. Micromegakaryocyte, mononuclear megakaryocytes, and hyperlobulated megakaryocytes are important diagnostic features of MDS and are reliable morphological findings of dysplasia. In the bone marrow the megakaryocytes may be clustered or adjacent to the bony trabecula. The del 5q syndrome has mononuclear megakaryocytes that are of normal size but with a single eccentrically placed round non-lobulated nucleus [3]. Megaloblastic changes (nuclear cytoplasm asynchrony) can be seen in the myeloid and erythroid precursors. Dysgranulopoiesis and dyserythropoiesis are more readily noted in the bone marrow aspirate smear and not the biopsy. The bone marrow smear is necessary to identify ring sideroblasts that may not be apparent on the biopsy sample. Immunohistochemistry may be a useful supplement to histology. Small mononuclear megakaryocytes can be confused with myeloid precursors. A biopsy is necessary to access the degree of reticulin fibrosis and overall bone marrow cellularity. Immunophenotyping using flow cytometry on the bone marrow and/or peripheral blood may be helpful in the diagnosis and defining prognosis and response to treatment. However, while controversial there are currently no accepted standards for the diagnosis of MDS by flow cytometry.

The finding of aberrant immunophenotyping of myeloid blasts is helpful in corroborating the diagnosis of MDS, but is not diagnostic of MDS. The aberrant expression of the lymphoid antigen CD7 on myeloid blasts is a common phenotypic abnormality and correlates with a poor prognosis [42]. Increase and/or clustering of blasts favors MDS. Immunostaining for CD34 on core biopsy is very helpful to estimate blast numbers and possible clustering. In the absence of reliable aspirate smear, CD34 immunostaining on core biopsy and/or clot section can be used for estimating percentage of blasts. The use of flow cytometry on both the PB and BM is the focus of many studies and should be part of the initial evaluation of MDS [42].

Clinical and Prognostic Features

The initial evaluation of all patients with MDS should be performed before planning treatment and should include a detailed history of prior exposures to chemotherapy, radiation therapy or toxic exposures. The cellularity should be noted from the bone marrow biopsy. The percent of blasts and the iron stain and the presence of ring sideroblasts should be performed on the bone marrow aspirate. Iron studies including a ferritin and transferrin saturation should be obtained prior to starting growth factors and on patients who are receiving red cell transfusions. A serum erythropoietin should be determined in patients with symptomatic anemia. In patients who are candidates for an allogeneic hematopoietic stem cell transplant HLA typing should be performed on the patient and their siblings.

The WHO classification system attempts to offer general prognostic guidance for each subtype but additional information is usually needed to assign prognosis and plan therapy. In an effort to determine prognosis a number of prognostic scoring systems have been developed including the International Prognostic Scoring System (IPSS), the MD Anderson Prognostic Scoring System (MDAPSS), World Health Organization –based Prognostic Scoring System (WPSS) and others models have been developed to define the prognosis and guide therapy [69, 70]. The widely used and generally accepted International Prognostic Scoring System (IPSS) developed in 1997 and recently revised, IPSS-R, addresses clinical features not included in the WHO classification and attempts to define prognosis and leukemic progression. The IPSS-R included 5 cytogenetic subsets reflecting the importance of new prognostically important cytogenetic groupings (Table 23.9). The scoring system assigns a point score for each the following variables: the number of bone marrow blast forms, karyotypic abnormalities, and number of cell lines affected (cytopenias) (Table 23.10). The combined score determines the overall risk category: very low (risk score $\leq$ 1.50), Low (risk score > 1.5–3.0), Intermediate (risk score > 3–4.5), High (risk score > 4.5–6) and very high (risk score > 6). The IPSS-R risk category and score correlates with the overall survival and probability of transformation to AML (Table 23.9).

The other prognostic scoring systems include similar parameters but the IPSS-R scoring system continues to be widely used for stratification of patients enrolled in clinical trials [69, 70]. However, the IPPS-R system has a number of important limitations. The IPPS-R system is based in part on the FAB classification of MDS and includes MDS patients with 30% blasts. The threshold for AML in the WHO classification is 20% blasts which is not reflected in the IPSS–R blast scoring system. The IPSS also does not completely address the severity of the cytopenias or the need for transfusion support and does not take into account other prognostic

Table 23.9 MDS cytogenetic scoring system: IPSS-R

Prognostic subgroup	Cytogenetic abnormalities	Percent of patients	Survival (years median)	AML evolution[a]
Very good	−Y, del(11q)	4	5.4	NR
Good	Normal, del(5q), del(12p), del(20q), double including del(5q)	72	4.8	9.4
Intermediate	Del(7q), +8, +19, i(17q) any other single or double independent clones	13	2.7	2.5
Poor	−7, inv.(3)/t(3q)/del(3q), double including- 7/del(7q),complex:3 abnormalities.	4	1.5	1.7
Very poor	Complex: >3 abnormalities	7	0.7	0.7

NR not reached
[a]AML evolution 25%—median time to 25% AML: median—years

Table 23.10 IPSS-R scoring system

Prog score variable	0	0.5	1.0	1.5	2.0	3.0	4.0
Cytogenetics	Very good		Good		Intermediate	Poor	Very poor
Bone marrow %blasts	≤2		>2–<5		5–10	>10	
Hemoglobin g/dL	≥10		8–<10	<8			
Platelets cmm	≥100	50–100	<50				
Absolute neutrophil counts/cmm	≥0.8	<0.8					

Very Good −7,del(11q), Good—Normal, del(5q),del(12p), del (20q), double including del(5q) Intermediate - del(7q), +8,+19,i(17q), any other single or double independentclones. Poor—7, inv.(3)/t3q)/del 3, double including −7/del(7q). Very Poor—Complex >3 abnormalities. See Table 23.3

variables. The IPSS-R acknowledged the prognostic importance of additional variable but did not assign a point score to these factors including LDH, serum ferritin, β2-microglobulin, marrow fibrosis, patient's age, performance status and comorbidities. Other prognostic factors including disease duration or prior treatments are not part of the IPSS-R. The IPPS and IPSS-R was intended to assign prognosis at the time of diagnosis and therefore is a static score that was not intended to change with time or treatments. The IPSS–R also includes a number of uncommon cytogenetic subsets does not address the expanding role of molecular genetic studies in MDS.

MDS is often broadly separated, for treatment decisions, into low risk and high-risk disease based on overall survival and risk of AML transformation. The lower risk subtypes include MDS with single lineage dysplasia, MDS with Multilineage dysplasia, MDS with ring sideroblasts, MDS with isolated del(5q). The IPSS-R low risk categories include the very low, low and intermediate risk categories. These groups are associated with a general survival of >3 years and a low risk for transformation to AML and generally correspond to a IPPS-R score of <3.5. In contrast the higher risk MDSs groups include MDS-EB 1 and 2 and IPSS-R groups high and very high and are associated with a greater risk for transformation to AML [70].

Treatment

The treatment of a patient with MDS should be individualized based on the patient's age, subtype, IPSS-R risk category, performance status, cytogenetics and co-morbid medical problems. The majority of patients with MDS are elderly and tolerate intensive chemotherapy poorly. Moreover, standard therapies do not result in a cure and their impact on survival for most patients is unclear. Therefore any potential benefits of treatment must be weighed against the side effects and the patient's overall prognosis. The alleviation of disease-related complications and improved quality of life are important goals for most patients. The most appropriate care for many patients still remains supportive care. Although there are a number of therapeutic options available for MDS patients, none, other than an allogeneic stem cell transplantation, offers the potential for cure. The therapeutic options for patients with MDS include the use of hematopoietic growth and trophic factors, immunosuppressive agents, low-intensity cytoreductive chemotherapy including the hypomethylating agents, and intensive chemotherapy. While advances in the diagnosis and risk stratification has refined the prognosis for patients and defined gene mutations that are potential targets in MDS, no new drugs s have been approved for the treatment of MDS in over a decade.

Guidelines for evaluating the response to treatment in patients with MDS have been updated and incorporated into the criteria by the International Working Group (IWG) [71]. These guidelines attempted to define standard, criteria for complete and partial responses to treatment. Moreover, the response criteria emphasized that the goals of treatment of MDS is to alter the natural history of the disease and alleviate the disease-related complications and improve the quality

of life. Stable disease or minimal responses are difficult to interpret and make comparisons between trials difficult. In addition, the response rate in some Phase II trials did not translate into prolongation of survival, time to treatment failure, or improvement in the quality of life. The IWG criteria are a useful standard to use for comparing results across therapeutic trials and are now widely used for defining response to treatment.

Supportive Therapy

In many patients the diagnosis of MDS may require a period of observation and reevaluation. The WHO appropriately noted that in some patients reevaluating the peripheral blood and bone marrow after 3–6 months period was essential to exclude other causes of the dysplasia. In patients with indolent disease or who are asymptomatic, elderly, and frail or have co-morbidities, supportive therapy including transfusions represents a widely accepted standard of care. Patients should be followed for a change in their clinical pattern i.e., increase in red cell transfusion, declining platelet count, circulating blast forms, splenomegaly or decline in performance status. Red cell and platelet transfusions are administered for the symptomatic treatment of the anemia and thrombocytopenia. There is no one single hemoglobin cut off at which RBC transfusion should be offered to all patients but the use of transfusion support is increasing over the years in an effort to maintain a higher hemoglobin/hematocrit. Platelet transfusions are generally given when the platelet count is $<10{,}000 \times 10^9$/L but should be adjusted on the basis of individual risk factors and bleeding history. Thrombocytopenia is common in MDS and bleeding complications are exacerbated by impaired platelet function [68]. Platelet dysfunction is common and patients may bleed even with an adequate platelet count. Therefore, platelet support may be required prior to surgery and procedures to prevent excess bleeding. Patients with a platelet count of $\leq 20{,}000 \times 10^9$/L are at higher risk for bleeding. Disease modifying agents such a lenalidomide and hypomethylating agents are associated with thrombocytopenia. The repeated use of platelet transfusions is associated with allo immunization and transfusion reactions. Danazol an attenuated synthetic androgen with immune modulating activity may be effect in some thrombocytopenic patients with MDS [72]. Thrombopoietin receptor agonist (TPO) are being tested in clinical trials as single agents in low-risk MDS patients and in combination therapy with disease modifying agents (lenalidomide) in high risk MDS. Romiplostim a Fc- peptide fusion protein with no sequence homology with endogenous TPO has been evaluated in low /intermediate risk MDS patients with thrombocytopenia [73, 74]. Romiplostim reduced overall bleeding events but the trial was stopped because of concerns regarding leukemic transformation. Eltrombopag and oral nonpeptide, noncompetitive TPO receptor agonist which is indicated for the treatment of ITP was evaluated in a phase 2 randomized trial in low/intermediate risk MDS patients, Eltrombopag increased the platelet count in a limited number of patients without a increase in leukemic risk. It was unclear in the limited studies that either TPO improved survival. The TPOs are not approved at this time for treatment of thrombocytopenia in MDS patients.

Neutropenia and impaired neutrophil function are also common in MDS patients. The use of prophylactic antibiotics, however, is not warranted for most patients. Neutropenia without a history of recurrent infection is not a justification for the initiation of therapy. Granulocyte colony stimulating factor or granulocyte macrophage colony stimulating factors can transiently increase the neutrophil and blast count in many patient with MDS. However, the clinical benefit of these growth factors is unclear. The use of G-CSF did decrease the incidence of serious infections but did not favorably impact survival in a prospective controlled trial. The use of these cytokines did increase the white blood count and the number of circulating blasts but did not appear to accelerate the progression to acute leukemia. Although in selected patients with active, serious infections there may be a role for the use of these cytokines in the MDS patient with neutropenia in combination with antibiotics, at present there is no evidence to support the general use of either G-CSF or GM-CSF [74].

Patients may require multiple transfusions over many years and the potential for iron overload should be addressed early in a patient's course. Each unit of RBC contains 200–250 mg of iron and iron overload from transfusions occurs when a patient has received 25 units of packed red cells [75–79]. The benefits of chelation therapy (ICT) in MDS remains controversial. Patients with transfusion dependent low risk MDS may benefit from the early introduction of iron chelation and chelation may reduce the effects of iron overload on cardiac and possibly prevent end organ damage due to tissue iron overload [77]. Iron chelation may also reduce the risk of infections and improve survival after allogeneic hematopoietic stem cell transplantation and may delay the leukemic progression and improve hematopoiesis [78]. Some observations suggest that cytopenias in iron-overloaded patients with MDS could be mitigated by ICT, possibly by a decrease in reactive oxygen species-mediated damage to hematopoietic cells [79]. In addition hematologic improvement was seen in some patients who received deferasirox including a normalization of labile plasma iron [79]. The use of ICT may suppress ineffective erythropoiesis by reducing iron and/or oxidative stress and modulating proliferation and differentiation. However, the risk of iron overload in transfusion dependent MDS patients is unclear. Iron toxicity in MDS patients may not only depend on tissue iron accumulation but also the extent of non-transferrin bound iron. Prospective studies evaluating the clinical benefit of iron chelation in MDS patients ongoing [77]. Iron chelation is a slow process and

therefore it is important to view chelation as a preventive supportive measure. The use of deferoxamine is impractical for most patients with MDS. Deferoxamine has a short half-life and requires a prolonged parental, either subcutaneous or intravenous, infusion that is administered over 8–12 h. The rapid infusion of deferoxamine following a red cell transfusion has limited benefit and does not result effective chelation. The oral iron chelator deferasirox (Exjade, Jadenu) is administered daily and is effective in reducing the serum ferritin [79]. Deferasirox can cause reversible renal insufficiency and GI disturbances, including gastrointestinal bleeding. In a number of studies Deferasirox was poorly tolerated in MDS patients with serious renal and gastrointestinal side effects [76]. A number of guidelines on the use of chelation therapy in MDS have been reviewed and a consensus statement published recommending the use of oral chelation therapy in transfusion dependent patients with low risk MDS. Iron overload may contribute to increased morbidity and mortality in low risk MDS patients. However other studies did not demonstrate a direct correlation of the serum ferritin, numbers of transfusions and overall survival. The benefits of prolonged oral chelation therapy is low risk MDS patients remains controversial and in general the benefits should be weighed against the potential risks and its effect on quality of life. The beneficial effects of iron chelation therapy on organ function and survival in transfusion dependent MDS patients is currently lacking. But, in low risk transfusion dependent patients with a ferritin of >1000 mg/dL or patients considered eligible for an allogeneic stem cell transplant ICT should be considered and renal and hepatic function closely monitored.

Erythropoiesis Stimulating Agents (ESAs)

Recombinant hematopoietic growth factors have been used with varying degrees of success to treat the cytopenias in MDS. Recombinant human erythropoietin (rHuEPO, EPO and darbepoetin), granulocyte-colony-stimulating factor (G-CSF), and granulocyte-macrophage colony stimulating factor (GM-CSF) have a role in managing the anemia and neutropenia in selected patients with MDS. ESAs have been studied extensively and approximately 30% of anemic patients with MDS will respond to treatment [75]. The reported response rates in low risk MDS varies between 30 and 82% depending on patient selection and response criteria [80]. The best responses to erythropoiesis stimulating agents (ESA) are observed in patients with an endogenous erythropoietin level of <500 U, a transfusion requirement of less than two units of red cells a months and low risk MDS with <5% myeloblasts [81]. ESAs decrease red cell transfusion requirements and improved the quality of life (QOL). Higher doses of EPO appear to enhance the erythroid responses. The combination of EPO plus G-CSF appears to be synergistic and may optimize the response to EPO. The duration of response was 11–24 months and EPO with or without G-CSF did not increase the incidence of AML transformation. ESAs did improve the QOL but treatment did not impact on overall survival. The ESAs have been associated with an increased mortality, possible promotion of tumor growth in solid tumors and thromboembolic events in non-MDS patients. No increase in treatment related either cardiovascular or thrombotic events occurred in patients who received either EPO alone or combination cytokines EPO with G-CSF as compared to a control population. The FDA recently cited safety concerns from data in clinical studies with ESAs administered to patients with solid tumors [81]. In patients with various solid tumors the use of ESAs was associated with a shortened survival, and/or increased risk of tumor progression or recurrence as well as a increased risk for thrombotic events in patients with renal disease (http://www.fdagov/Drugs/DrugSafety/). MDS patients did not have a increase in disease progression or thrombotic complications associated with ESAs [80]. A predictive model for treatment with EPO and G-CSF demonstrated that patients with an EPO level of <500 mU/mL, and a pretreatment transfusion requirement of less than 2 units/month responded best to ESAs [81]. Best responses were noted in IPSS lower risk patients. Responses can take 8 or more weeks with most patients responding by 12 weeks and the recommended starting for the recombinant human erythropoietin alpha (rEPO) doses are 40–60,000 units administered once or twice a weekly, and increasing the dose up to 300,000 U/week depending on the response. The longer acting form, Darbepoetin, is administered starting at 50–300 mcg/weekly or every other week. Darpoetin administered every 2–3 weeks at a dose of 500 mcg is also effective. Adequate iron stores should be documented prior to starting EPO treatment and during therapy as the failure to respond or loss of response may be a manifestation of depleted iron stores. Low doses of G-CSF (1 μg/kg) with EPO can be added to patients who fail to respond and appeared to augment the response in selected patients [80]. The mechanism of the response to high doses of EPO is unclear but the growth factors may modulate apoptosis in MDS progenitors and enhance erythropoiesis. Moreover, the response to ESAs may define a more favorable group of MDS patients and in a Phase III trial MDS patients who responded to EPO had a longer overall survival versus those who did not respond [80].

Thrombocytopenia is an independent adverse prognostic factor for survival in MDS. Bleeding complications resulting from thrombocytopenia and MDS associated platelet dysfunction are major causes of morbidity and mortality in MDS. Treatments to increase the platelet count are limited. Danazol is an attenuated, synthetic androgen that has been used in the treatment of immune-mediated thrombocytopenia (ITP). Danazol may increase the platelet count in low-risk MDS patients who are thrombocytopenic [72]. The mechanism

of action is unclear, but may reflect that some patients with MDS have immune-mediated thrombocytopenia that responds to the immunoregulatory effects of danazol. Danazol, 200 mg po tid, is generally well tolerated and associated with an increase in platelets in 10–46% of treated patients. The duration of response is variable, 2–26+ months, and maintained only while the drug is administered. Patients with platelet counts greater than 15×10^9/L appear to respond best, and the impact on survival or disease progression is unknown. Two thrombopoietin receptor agonists (TPO), eltrombopag and romiplostim, are currently available to treat patient with refractory ITP. Both agents are active in patients with ITP by increasing platelet production. However, safety concerns remain including the risk of marrow fibrosis and leukemic transformation [68]. A transient increase in blasts forms has been noted in patients treated with TPO. Eltrombopag is currently being studied in higher risk MDS patients. Ongoing studies are evaluating the role of these agents in the treatment of the thrombocytopenic patient with MDS. These early trials of TPOs suggest that they are well tolerated in MDS patients and increased the platelet count and decreased the need for platelet transfusions and clinical bleeding events. But their effect on survival and disease progression is unclear.

Immunosuppressive Therapy

Patients with MDS can present with a number of immune-mediating pancytopenias that potentially respond to immunosuppressive therapy [82]. Some patients with low risk and hypocellular MDS responded to immunosuppressive regimens used for the treatment of aplastic anemia. Selected patients with hypocellular or normal cellular bone marrows responded to the administration of anti-thymocyte globulin (ATG), cyclosporine A (CSA) and steroids [83]. Antithymocyte globulin (ATG) either rabbit or hoarse derived, administered at doses similar to those used in the treatment of aplastic anemia resulted in improvement of one or more cytopenia in 30–50% of selected patients [83]. Trilineage responses were observed in some patients and the median duration of response was 10+ months. Responses were more frequent with hypocellular MDS but were even noted in patients with normocellular bone marrows. Improvements were seen in patients with low-, intermediate-, and high-risk IPSS scores. Age, 60< years, HLA- DR15 positivity, low risk disease, shorter duration of transfusion dependency, and trisomy 8 cytogenetic abnormality correlated with the response to treatment [84]. The responses did not correlate with the loss of a previously noted cytogenetic abnormality, suggesting that the treatment was not affecting the MDS clone. A majority of the patients attained a partial response and treatment did not restore normal hematopoiesis. The mechanism of action is not clear but in vitro studies suggest that the response may be mediated by a loss of cytotoxic T-lymphocyte activity which correlated with changes in the T-cell receptor profiles [84]. The response to ATG appears greatest in low-risk patients. In some patients who initially responded to ATG and then relapsed, retreatment was effective. Toxicity of ATG therapy included fevers, infusion related side effects, and infections. Age was the most important predictor of response with patients >60 years of age having a poorer outcome and increased complications associated with immunosuppressive therapy. These studies suggested that older patients with MDS may have a decreased marrow reserve associated with a diminished response to immune-suppression. CSA, alone may also be effective in hypocellular MDS patients. The combination of CSA and ATG may enhance the response in selected patients. In addition the use of alemtuzumab, an antibody against CD52, has been used in selected patients. The reported studies of ATG and CSA treated a highly selected subset of patients that represent a minority of patients with MDS. Most of the responding patients presented with hypocellular marrows (<15% cellularity) with minimal dysplasia. The responses of MDS patients to immunosuppressive therapy reflect the heterogeneity of the disorder and the relationship between the immune system, marrow suppression and MDS remains controversial. In addition it is unclear if the use of ATG with or with CSA impacted overall survival. In at least one Phase 3 trial comparing ATG + CSA versus best supportive care found no difference in overall survival or transformation to AML in the ATG + CSA arm. The use of immunosuppressive therapy to modulate the abnormal clone represents a controversial but a potential treatment that needs to be further evaluated to define the optimal group of patients who are candidates for immunosuppression.

Lenalidomide

Lenalidomide is a second generation 4-amino-gluteramide analog of thalidomide. Lenalidomide has multiple modes of action including pro-apoptotic cytokine generation, T cell stimulation or inhibition, antiangiogenesis, altering cell adhesions to bone marrow stroma, direct antiproliferative activity and the inhibition of a pro-inflammatory cytokines [85]. Lenalidomide was active in patients with del (5q) by selectively inhibiting the del(5q) clone. Lenalidomide in a phase II trial was administered at 10 mg/day for a 28 cycle or 21/28 days cycle in transfusion dependent patients with del(5q) abnormality 67% of patients became transfusion independent and 45% obtained a complete cytogenetic; complete and partial responses occurred in 84% of patients with del (5q) [63]. The finding of additional cytogenetic abnormalities, with the exception of −7/del(7q), did not significantly affect the response to lenalidomide. All patients who had a cytogenetic response became transfusion independent which was associated with improvement in overall survival. Patients who did not response to lenalidomide had higher rate of progression to AML. The median duration of response was 2.2 years (range 0.1–4.4 years)

and approximately 30% of patients remain transfusion independent after 3 years [63]. Most of the responding patients developed pancytopenia and the myelosuppression served as a surrogate marker of clonal suppression of the del(5q) clone and predictive of the response to lenalidomide. Greater than 70% of patients with the del(5q) developed thrombocytopenia within the first 4–8 weeks of treatment and the development of thrombocytopenia was significantly correlated with a favorable response and the development of transfusion independence and a cytogenetic response [63]. A platelet decline in the first 8 weeks of treatment correlated with the response to treatment and likely reflected a direct specific cytotoxic effect of lenalidomide on the MDS clone. Most patients needed dose reductions due to myelosuppression and/or thrombocytopenia and in phase III trial comparing 5 daily and 10 mg on days 1–21 on a 28-day cycle versus placebo, the 10 mg dose was superior. Long term follow up in patients treated with lenalidomide demonstrated a prolonged duration of response, with durable transfusion independence, cytogenetic responses and extended survival with improvement in quality of life [63]. In low risk, transfusion dependent MDS patient without the del (5q) cytogenetic abnormality the response rate to lenalidomide was much less and the mechanism of action appears to be very different. The overall response rate in this non del(5q) group was 33% but only 17% became transfusion independent. A majority of the responses were characterized as a decrease in RBC transfusions as compared to the baseline transfusion requirement. There were minimal or no changes in the bone marrow morphology and documented histological and cytogenetic responses were rare. Myelosuppression, grade 3/4, neutropenia and thrombocytopenia occurred only in 20–25% of patients. Moreover, the development of cytopenias including thrombocytopenia and neutropenia on lenalidomide for patients without the del(5q) was not associated with a response to treatment. The response duration of lenalidomide in the non-del (5q) patients ranged from 3 to 85 weeks with a median of 41 weeks. The median time to response was short at 4.8 weeks and response after 16 weeks of treatment were rare and alternate treatment was recommended for patients who fail to respond after 4 months of lenalidomide. Higher responses correlated with a baseline platelet count of $>150 \times 10^9$/L and shorter duration of MDS. Lenalidomide mechanism of action in MDS is clearly karyotype dependent. In del(5q) patient's lenalidomide appears to have a direct cytotoxic effect on the dysplastic clone resulting in eradication of the malignant clone. In contrast in the lower risk transfusion dependent MDs patient without the del(5q) lenalidomide effects appear to be mediated indirectly perhaps by effecting the bone marrow microenvironment or cytokine modulation.

Low-Dose Chemotherapy

Potential Differentiating Agents

Cytarabine administered at low doses (low dose Ara-C: Lo DAC) has been the most extensively used chemotherapy for elderly patients with high-risk, symptomatic MDS [86]. Cytarabine is administered daily by either continuous infusion or bolus subcutaneous injections at doses of 10–20/mg/m^2 for 14–21 days. At low dose cytarabine may induce differentiation but most likely works by a direct cytotoxicity [86]. In a prospective controlled trial patients higher risk MDS patients had the highest response rates, 20–35% (complete remission partial response [CR] + PR). The median duration of response for all subtypes was 8–15 months, with a range of 6–24 months. The response to Lo DAC correlated with its cytoreductive effect on the bone marrow. In a Phase III trial treatment with low-dose cytarabine was not superior to supportive care with regards to overall survival or leukemic transformation [86]. The role of low-dose cytarabine in the treatment of patients with high-risk MDS is controversial. In high-risk patients it remains a widely used treatment that may be effective in inducing transient responses in a subset of patients with progressive disease who are not candidates for alternative treatments. Low doses of chemotherapy may have a supportive role in selected patients with MDS. A number of trials have addressed the use of low doses of oral chemotherapy that was well tolerated and administered over a prolonged period. Low-dose oral etoposide (VP-16) and hydroxyurea were effective in controlling symptoms in some patients [87]. The use of steroids alleviated some of the cytokine-mediated symptoms associated with MDS/MPN and produced transient hematological improvements. Low-dose melphalan (2 mg/day until response or progression) in high-risk, frail patients with MDS was well tolerated and responses were noted in both hypercellular and hypocellular MDS [88]. The overall response rate to a prolonged course of low-dose melphalan in Phase II studies was approximately 40%. The duration of the responses to low-dose melphalan is unclear and additional studies are needed to determine its role in treating high risk MDS.

Histone acetylation facilitates active gene transcription and highly regulated by histone deacylases (HDACs) and histone acetyltransferases (HATs). HDAC inhibition may restore normal acetylations of histone proteins and promote cell cycle arrest and induce apoptosis in malignant cells. Normal differentiation and cell death programs are influenced by histone modification. HDAC expression is frequently deregulated in high risk MDS and AML and is therefore a potential therapeutic target. A number of HDAC inhibitors have been developed including Valproic acid, Entinostat, Belinostat, Panobinostat, Romidepsin, Vorinostat and others in various stages of development. HDAC inhibitors have multiple potential mechanism of action leading to

their pleiotropic activity and use in various disorders. The response rate for single agent HDACs is low and therefore they are combined with other agents including the DNA methyltransferase inhibitors including decitabine and azacitidine [88]. The potential synergism between demethylation and histone deacetylase inhibition made the use of combination therapy the focus of a number of ongoing trials.

DNA Methyltransferase Inhibitors

The DNA hypomethylating agents, 5-azacytidine (Azacitidine—Vidaza) and 2,5-deoxycytidine (Decitabine—Dacogen) are both analogs and the pyrimidine nucleoside cytidine. Both drugs inhibit DNA methyltransferase, reduce DNA methylation, and may induce re-expression of key tumor suppressor genes in MDS [89]. They are incorporated into the DNA of cells and result in hypomethylation of critical residues, cytosine prior to guanine sequences, (CpG) in the promoter regions in the DNA. At low doses they are believed to induce hypomethylation through depletion of cellular DNA methyltransferases and at higher doses both agents are cytotoxic by incorporation into RNA and/or DNA. The use of low doses in MDS is based on the principle that hypomethylation of DNA leads to reactivation of tumor suppressor genes expression which is passed down through subsequent generation of MDS cells.

The effect of azacitidine was evaluated in a randomized phase III trial. Azacitidine-treated patients showed a better overall response compared to those treated with supportive care only (60% versus 5%) and a longer time to progression to AML or death, but no overall survival advantage. A confirmatory international phase III trial evaluating the effects on long-term outcome with azacitidine versus conventional care (i.e., physician choice of low-dose cytarabine, standard chemotherapy, or best supportive care). Azacitidine was administered subcutaneously (75 g/m^2/day) for seven consecutive days every 28 days for patients with high risk MDS. Azacitidine treatment was associated with a significantly median overall survival 24.4 months compared to 15 months in the conventional care arm [89]. Most patients in the conventional treatment arm received low dose cytarabine. The 2 year survival was 50.8% in azacitidine as compared with 26.2% in the control, conventional treatment arm. This was the first randomized study to demonstrate a survival advantage for treatment with a hypomethylating agent. While some patients had a complete clinical response while receiving Azacitidine the durations of the complete response were short and not maintained when treatment was stopped. Myelosuppression was frequently observed in patients receiving Azacitidine and its role as a differentiating agent or de repressor of tumor suppressor genes in MDS was unclear. Responses were associated and improved quality of life in some patients. But in other trials the impact of treatment on survival was unclear. An oral formulation of 5-azacitidine is being studied in patients with low risk—intermediate risk MDS.

Decitabine (DAC) is a more potent inhibitor of DNA methyltransferase than azacitidine and is associated with greater myelosuppression. Decitabine has been effective in patients with high-risk MDS and may prolong the time to transformation to AML. The schedule of decitabine administration suggests that both the duration, dose and number of doses are important. Decitabine is given intravenously at various doses and dose schedules. The Phase I, II, and early Phase III trials DAC was administered the dose was 15 mg/m^2 over 3–4 h every 8 h for 3 days. The overall response rate in MDS was 49 and 64% in high-risk patients. The actuarial median survival time was 15 months and myelosuppression was common. In a randomized dose finding study, DAC was administered at 20 mg/m^2 IV over 1 h daily for 5 days; while not the FDA approved dosing, it is the standard of care at many institutions [89]. The CR rate in a dose finding trial was 39% and an overall response rate of 70%. The CR rate of 34% versus 9% when decitabine was given at a higher dose with fewer and less frequent cycles. Different dosing schedule have been associated with less myelosuppression and similar efficacy. Decitabine administered weekly, or 3 days a week was associated with a 60% trilineage response with minimal hematologic toxicity [90].

Phase III randomized studies however, did not demonstrate a survival benefit with DAC in a very high risk patient population. Azacitidine and decitabine generally require 3–6 treatment cycles to obtain an optimal therapeutic response, suggesting that the mechanism of action is more than just cytoreduction. For high-risk MDS, the hypomethylating agents either azacitidine and decitabine continue to be the treatments of choice [88]. MDS patients with monosomy 7(del 7) either alone or with other complex cytogenetic abnormalities may be particularly sensitive to treatment with DNMT inhibitors. High cytogenetic responses rates have been reported with both azacitidine and decitabine in retrospective analysis and both agents appear to alter the natural course of MDS and may be of benefit for selected patients. However, additional studies are needed to define the duration of therapy, dosing and their role as single agents or in combination for high risk patients with MDS. Some trials have shown a correlation between expression of methylated genes and clinical response but this finding remains controversial. Early trials using a combination of azacitidine and lenalidomide have reported acceptable toxicity and encouraging activity albeit in limited number of selected patients. In patients with low risk disease who have failed growth factors and/or lenalidomide the use of one of low dose azacitine or decitabine is associated with decrease in transfusion requirement with acceptable toxicity.

Intensive Chemotherapy

Combination, intensive cytoreductive induction chemotherapy regimens result in meaningful toxicity and modes

responses in patients with high risk MDS. The complete remission rate with intensive AML regimens is 40–60% with a 20–30% induction related mortality [88]. High risk cytogenetics, advanced age, and performance status are associated with poor response to intensive chemotherapy. In a retrospective study evaluating 510 patients with high risk MDS who received intensive chemotherapy the induction related mortality was 17% and 5 year survival probability 8% [37]. However in selected younger patients who present with poor prognosis MDS and who may proceed to an allogeneic transplant are potential candidates for intensive combination chemotherapy. Newly diagnosed patients with high risk MDS without a prior history of MDS appear to respond to standard AML induction chemotherapy in a similar fashion to de novo AML. However, patients who present with an antecedent or evolving MDS generally respond poorly to intensive AML-type induction chemotherapy [88]. The complete remission rate is 13–40% with an incidence of toxic deaths during therapy of 1–53%. Moreover, the role of post induction chemotherapy is unclear in patients with MDS who respond to treatment. In selected younger patients with MDS intensive chemotherapy including an anthracycline and cytarabine has resulted in complete remission rates of 20–60% but with a relapse rate of 90%. The remission durations are typically 6–12 months with only rare instances of prolonged disease-free survival. The poor response of MDS patients to intensive induction chemotherapy reflects the biological differences between de novo AML that occurs in younger patients and MDS. Moreover, in patients who have poor risk cytogenetics and prior MDS treatment or a longer time to progression responded poorly to induction chemotherapy with a median overall survival of less than 4 months. Moreover a CR may not impact overall survival in patients with MDS and therefore may not be the best end point for evaluating studies. The use of AML intensive induction regimens should be reserved selected younger patients with high risk disease as a bridge to an allogeneic stem cell transplant or as part of a investigational trial.

Stem Cell Transplantation

Autologous and allogeneic stem cell transplantation has been used in patients with MDS or MDS-AML. The role of autologous transplantation remains very controversial. The 3-year disease-free survival in a selected group of patients ranges from 14 to 58% in single arm or single institution studies [90]. All patients were transplanted in first remission and adequate number of stem cells were harvested. The 2-year survival in selected patients reported to the EBMT for patients in first complete remission was 39%, with a disease-free survival of 34% and a relapse rate of 64% [90]. In patients over the age of 40 years the disease-free survival was 25%. Patients younger than 40 years of age, and in first complete remission, responded better. The transplant-related mortality was high: 39% and 17% for patients over and younger than the age of 40 years, respectively. The relapse rate is higher than in patients with AML. Larger prospective studies are needed to define the potential role, if any, of an autologous stem cell transplantation for patients with MDS. However it is possible, in selected patients, to harvest polyclonal and karyotypically normal progenitors from the peripheral blood in younger patients with MDS following intensive induction chemotherapy [90].

Allogeneic stem cell transplantation represents the only potential curative therapy for patients with MDS and should be considered for patients who have an HLA compatible donor. While there are no prospective randomized trials that have compared outcomes of HSCT versus no HSCT for patients with MDS who have eligible donors it is accepted as the standard of care and generally recommended for selected low risk and all clinical eligible high risk patients with MDS [91]. However, patients with MDS have an increased incidence of transplant-related mortality as compared to patients with AML undergoing similar HSCTs [91]. The non relapse morbidity and mortality in MDS patients is high and not solely explained by the older age of the patient population [91]. Younger patients with MDS also appear to have an increased incidence of transplant-related complications. Patients with advanced disease and unfavorable cytogenetics have a higher probability of relapse and lower overall survival. The outcome is better for patients with a lower risk IPSS-R score, who are less transfusion dependent and a good performance status. Moreover, the standard risk factors that predict overall survival in MDS appear to affect the post-transplant outcome [92]. Performance status, comorbidities and fragility scores predicted overall survival [93]. Age was not the most important factor relating to transplant related morbidity and mortality [93]. The timing of the transplant for most patients remains controversial [94]. The factors that determine the overall survival and rates of relapse of an allogenic stem cell transplant include the percent of blasts, transfusion dependence, serum ferritin, comorbidities and pre transplant genetic profiles. The IPSS-R does not consider the prognostic variables associated with transplant related complications or survival but the IPSS-R score correlates with the HSCT outcome. In the IPSS-R scoring system the very high risk MDS patients had a only 10–14 months survival from the time of diagnosis and derived limited benefit from standard treatments [95]. Patients with high risk MDS should therefore be referred early in their course for consideration of an hematopoietic allogeneic stem cell transplant (HSCT) [90]. The role of HSCT in low risk MDS, while a accepted standard of care is controversial [91]. Patients with (very) low and intermediated risk IPSS-R scores with good performance status and poor, high risk features should be considered for an

allogeneic hematopoietic stem cell transplant. Low risk patients with poor risk cytogenectics, life threatening cytopenias and a transfusion requirement of ≥ units a month for 6 months should be considered for early referral to a transplant center. While the percent of bone marrow blasts did not significantly effect the overall survival cytoreductive therapy prior to HSCT is recommended for patients with ≥ bone marrow myeloblasts [96]. Patients with low risk disease have a better transplant related outcome than patients with more advanced disease [96]. However, treatment related complications is a major cause of morbidity and mortality after a HSCT transplant and must be considered in advising younger patients with low risk disease. Moreover older patients with multiple co-morbidities, very poor risk cytogenetic and mutational studies should not be offered a HSCT due to the very low chance of a successful HSCT. The patients pretransplant genetic profile and mutational findings are important predictors for overall survival and should be included in a decision to recommend a HSCT. TP 53, RAS and JAK 2 mutations were associated with a signigficantly shorter overall survival following a HSCT. Mutational studies are important predictors of overall response and should be obtained on all patients prior to performing a HSCT [96]. The use of reduced intensity regimens have decreased the early transplant related mortality and are generally recommended for patients with co morbid conditions or older age. The reduced intensity regimens are associated with a decrease in non relapse mortality but a higher risk of relapse. The reduced intensity transplant depend on the immune effects mediated by the donor derived cells and graft versus disease effect. Reduced intensity transplants, however, have not impacted the overall incidence of acute and chronic GVHD which remains the major cause of morbidity and mortality in older patients. Chronic GVHD remains a serious and life long complication of HSCTs. Studies have documented the decrease in quality of life in older patients with chronic GVHD [97]. Chronic GVHD is associated with an increase in cardiovascular disease, metabolic syndrome, diabetes, cognitive decline, fatigue, sexual dysfunction and endocrine abnormalities. However, for the younger patient with a good performance status and a suitable HLA matched donor an allogeneic stem cell transplant should be considered as it represents the only potential curative therapy and should be considered earlier in high risk patients. Determining the optimal timing of the HSCT remains a controversial issue and is the subject of a number of ongoing clinical trials [97, 98].

Future Directions and Evolving Role of Molecular Genetics

Advances in treatment will parallel our understanding of the molecular and immunological events involved in development and progression of MDS. Molecular genetic technolology may help in defining the prognosis of patients with MDS [99]. In AML the use of mutational analysis has helped define the prognosis of patients with favorable or normal cytogenetics [100]. Going forward molecular genetics, including DNA array technology and whole genome sequencing (WGS) studies are likely to play a significant role in defining more biologically based prognostic scoring system. The current MDS prognosis scoring systems stratify untreated patients at the time of diagnosis. The scoring systems do not account for changes in the prognostically important covariables over time. Currently used scoring models predict outcome at diagnosis but do not predict overall survival and transformation over time. The need for a biologically based model of MDS that can reflect the heterogenous course of high and low risk patients is needed to better define therapy and evaluate long term trials. A recent large study of patients with MDS undergoing stem-cell transplantation and evaluating the association of mutations with transplantation outcomes identified subgroups of patients with prognostic and therapeutic (conditioning regimen) implications. So for example TP53 mutations in patients with MDS were associated with sorter survival and shorter time to relapse and so was presence of RAS pathway and JAK2 mutations. Knowing mutational status of these key genes along with other biological parameters will help in clinical decision making [101] (Table 23.10).

Aberrations in gene mutations are still poorly characterized in MDS. The most common point mutations in MDS are still not specific for MDS but are associated with secondary, prognostically important mutations. In addition, most cases of MDS are genetically heterogenous with a dominant clone and many subclones and based on whole genome sequencing studies (WGS) no single gene is exclusively mutated in the funding clone [102]. The combined use of flow cytometry, mutational analysis and DNA arrays may identify the most appropriate therapy for patients with MDS. A recent Genomic classification of AML defined specific genomic categories that are biologically and prognostically important [99, 100]. Hopefully a similar classification system can be developed from the large MDS data bases. Incorporating molecular genetic studies into diagnostic and prognostic models for MDS with help in defining the role of newer agents with precise targets. A biologically based system will help evaluate studies of combination with immune based treatments for different subsets of patients. While it is till unclear how somatic mutations will be applied in cases of MDS. MDS associated genes, however not specific, appear to be relevant clinical markers for the diagnosis and prognosis but have yet to be incorporated in prognostic scoring systems. More dynamic scoring systems are needed to define biologically important subtype. A molecular diagnostic system will facilitate comparing the results of different trials and assessing treatments to specific subtypes. Newer prognostic models that incorporate molecular genetic studies are

being developed and validated. The use of molecular genetic studies in a predictive model may provide for a more dynamic scoring system that evolves over the patient's course and may help in the developing targeted therapy and the timing of HSCTs [103].

Summary

The myelodysplastic syndromes are a heterogeneous group of disorders with a variable clinical course. The incidence of MDS is increasing with the aging population and now represents the most common hematologic malignancy in patients older than 60 years. A majority of MDS patients present with lower-risk MDS and do not progress to AML and die of infections or bleeding related to their MDS. The WHO classification system remains controversial but is currently accepted at the standard criteria for defining MDS and attempts to separate MDS from reactive disorders other clonal disorders. Defining and differentiating MDS from other clonal or reactive process is critical many disorders can result in dysplasia and cytopenias. Clonal cytogenetic abnormalities are helpful in establishing a diagnosis of MDS, particularly when morphologic findings are subtle. Multilineage dyspoiesis favors MDS; however, prominent dysplasia can be seen associated with chemotherapy, immunosuppressive medications, and nutritional deficiencies. The diagnosis and management of patients with MDS require the close cooperation of clinician, cytogenetist and pathologist. The application of newer molecular studies may aide in the diagnosis and planning therapy.

MDS diagnosis and classification is currently in a transitional phase from reliance almost entirely on cell morphology supplemented by cytochemistry and G-banded karyotyping, towards a new model in which molecular and perhaps immunophenotypic findings will be fully incorporated. The revised 2016 WHO MDS classification represents the new standard for the diagnosis of MDS and reflects the complexity and heterogeneity of MDS. The trend towards greater classification complexity seems likely as additional molecular and cytogenetic lesions in MDS are characterized and incorporated into the diagnosis and prognosis. It is hoped that with additional information unifying themes will emerge that will help in defining prognosis and address new therapies.

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Part III

Myeloma and Related Diseases

History of Multiple Myeloma

24

David P. Steensma and Robert A. Kyle

The Peculiar Case of Thomas Alexander McBean

Saturday, Nov. 1st 1845

> *Dear Dr. Jones,*
> *–The tube contains urine of very high specific gravity. When boiled it becomes slightly opaque. On the addition of nitric acid, it effervesces, assumes a reddish hue, and becomes quite clear; but as it cools, assumes the consistence and appearance which you see. Heat reliquifies it. What is it?* [1]

This cryptic note and a urine sample were sent by a leading London general practitioner, Dr. Thomas Watson, to Dr. Henry Bence Jones, a 31-year-old physician at St. George's Hospital who had already established a reputation as a skilled chemical pathologist [2].

The patient, Thomas Alexander McBean, was a 45-year-old successful London grocer of "temperate habits and exemplary conduct," who had been married since 1825 and had numerous children [3, 4]. With the exception of two or three severe attacks of "frontal neuralgia," Mr. McBean enjoyed good health prior to his final illness. Over the course of the year 1844, Mr. McBean's family noted that he fatigued easily and appeared to stoop while walking. He also developed urinary frequency, and grew concerned that "his body-linen was stiffened by his urine" despite the absence of a urethral discharge [4]. He took a countryside holiday in September 1844 to try to regain his strength, which he felt had been impaired by overwork and a family illness.

While vaulting out of an underground cavern on his rustic vacation, Mr. McBean "instantly felt as if something had snapped or given way within the chest, and for some minutes he lay in intense agony, unable to stir" [4]. After the intense pain subsided, the patient made his way to a nearby inn where he rested for the night, and he felt considerably better the following day.

Upon his return to London, Mr. McBean saw Dr. William Macintyre, a 53-year-old Harley Street consultant and physician to the Metropolitan Convalescent Institution and to the Western General Dispensary in St. Marylebone, for an opinion regarding dyspepsia. During his consultation with Dr. Macintyre, Mr. McBean mentioned his recent accident, from which he was still somewhat sore. Dr. Macintyre applied a strengthening plaster to the patient's chest to try to reduce the pain produced by arm movement. The plaster and avoidance of exertion allowed the patient to recover enough to resume his work as a grocer, although pain and stiffness of the chest persisted. A month later, Mr. McBean consulted with a surgeon when acute chest pain and shortness of breath recurred; the surgeon removed a pound of blood (about 450 mL), and applied leeches and blistering agents. This treatment did not relieve the symptoms and was followed by several months of weakness.

In the Spring of 1845, Mr. McBean developed right-sided pleuritic pain, which was treated by cupping. Additional therapeutic bleeding induced further weakness. Wasting, pallor, and slight puffiness of his face and ankles led the patient to consult with Dr. Watson, who prescribed steel and quinine therapy, commonly used at the time for asthenic patients who had mysterious illnesses, especially if fever was present. The patient rapidly improved. By the middle of the Summer of 1845, Mr. McBean was able to travel to Scotland on holiday, where on the seacoast "he was capable of taking active exercise on foot during the greater part of the day, bounding over the hills, to use his own expression, as nimbly as any of his companions" [4]. His appetite became ravenous—so much so that he dreamed of eating dogs and cats, and ate great quantities of fish [1, 4]. His recovery was interrupted by the onset of diarrhea, "which proved obstinate, and reduced his strength considerably" [4].

In September 1845, Mr. McBean returned to London in a debilitated state, but free of pain. However, in October,

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lumbar and sciatic pain developed and soon became severe. Warm baths, Dover's powder (ipecac and opium, as a purgative and for pain relief), tartrate of potash (frequently employed for rheumatism and for complex pain), acetate of ammonia (used in febrile and inflammatory conditions), camphor julep (frequently used for low-grade fevers, muscle spasms, and anxiety), and compound tincture of camphor did not help. The patient became confined to his home on Devonshire Street in the affluent Marylebone (Westminster) area of London. On October 30, 1845, Dr. Watson called in Dr. William Macintyre for a consultation.

Dr. Macintyre examined Mr. McBean's urine because of the patient's history of edema—a basic test that Dr. Watson had apparently neglected to perform. By 1845, urine examination was a well-established clinical practice, in large part because of the work of Richard Bright, a Guy's Hospital physician who linked proteinuria, "dropsy" (edema), and chronic kidney disease (thereafter known as "Bright's disease") in the 1820s [5]. Mr. McBean's urine specimen was opaque, acidic, and of high density, with a specific gravity of 1.035, but sugar was absent. When heated, the urine was found to "abound in animal matter"; with the addition of nitric acid, the turbid urine became clear, but a precipitate developed after an hour. Uniquely, this precipitate "underwent complete solution on the application of heat, but again consolidated on cooling" [1].

Upon receiving urine specimens sent by Drs. Watson and Macintyre on November 1, Bence Jones corroborated the finding that the addition of nitric acid produced a precipitate that was redissolved by heat and that formed again on cooling. He calculated that Mr. McBean was excreting more than 60 g/day of this proteinaceous material, and concluded that the protein was an oxide of albumin, specifically "hydrated deutoxide of albumen" [6]. Bence Jones calculated that there were 66.97 parts of "hydrated deutoxide of albumen" per 1000 parts of urine in the sample, and noted that this amount was equivalent to the proportion of albumin in healthy blood, so that every ounce of urine secreted was equivalent to the loss of protein from an equal volume of blood [1, 6].

Following Dr. Macintyre's visit, the patient continued to worsen. On November 3, 1845, the eminent clinical chemist Dr. William Prout (after whom Ernest Rutherford named the proton in 1920) joined Drs. Watson and Macintyre in consultation. Iron citrate and quinine were resumed, along with opiates, blistering agents, and counterirritants. Soon every movement of Mr. McBean's trunk produced excruciating pain. Great care and cautious maneuvering enabled the patient to "get in and out of bed on all fours," but he became weaker and eventually was confined to bed. He developed flatulence, and pronounced fullness and hardness in the region of the liver. He had phlegm in his chest and coughed fitfully, and suffered another episode of diarrhea.

On November 15, Dr. Bence Jones recommended treatment with alum, a substance used since ancient Egyptian times to clear turbid solutions, "with the view of checking the exhausting excretion of animal matter" [4]. The patient improved slightly in the following days and was able to sit up and enjoy his food, but on December 7 he "experienced a dreadful aggravation of lumbar pains" [4]. He had almost continual pain despite opiate treatment, became weaker, and died on January 1, 1846, exhausted but "in full possession of his mental faculties." The cause of death was listed as "atrophy from albuminuria" [3]. Involvement of the bones by the disease, aside from pain, was not recognized during the patient's illness.

Postmortem examination revealed emaciation. The ribs, which crumbled under the heel of the scalpel, were soft, brittle, readily broken, and easily cut by the knife. Their interior was filled with a soft "gelatiniform substance of a blood-red colour and unctuous feel" [4]. The sternum was soft and fragile and snapped when lifted. The heart and lungs were not remarkable. The liver was "voluminous, but of healthy structure." The kidneys appeared to be normal on both gross and microscopic examination, had "proved equal to the novel office assigned them," and were thought to have "discharged the task without sustaining, on their part, the slightest danger." The thoracic and lumbar vertebrae had the same degenerative changes as found in the ribs and sternum, but the humeri and femurs resisted "all efforts to bend or break them by manual force" [4].

John Dalrymple, surgeon to the Royal Ophthalmic Hospital, Moorfields (London), examined two lumbar vertebrae and a rib from Mr. McBean. In an 1846 paper on the "pathology of mollities ossium" (an obsolete general term for bony softness or fragility, analogous to the contemporary term "osteomalacia"), Mr. Dalrymple noted that the patient's lumbar vertebrae were markedly compressed and that they "scarcely exceed in thickness the intervertebral substance, and have lost nearly one-third of their normal bulk" [7]. The disease appeared to begin in the cancellous bone; it then grew and produced irregularly sized round dark red projections that were visible through the periosteum. Nucleated cells formed the bulk of the gelatiniform mass that filled the large cancellous cavities. Most of these cells were round or oval and about one-half to two times as large as an average blood cell. The cells contained one or two nuclei, each with a bright, distinct nucleolus. Wood engravings made from Mr. Dalrymple's drawings are consistent with the appearance of plasma cells (Fig. 24.1) [7]. Dalrymple postulated that these nucleated cells had a limited duration of life and then disintegrated, after which they were "carried out of the system by the circulation of the kidneys" [7].

In light of the autopsy findings, both Dalrymple and Macintyre believed that the disorder that Mr. McBean suffered from was a malignant disease of bone. Bright's disease

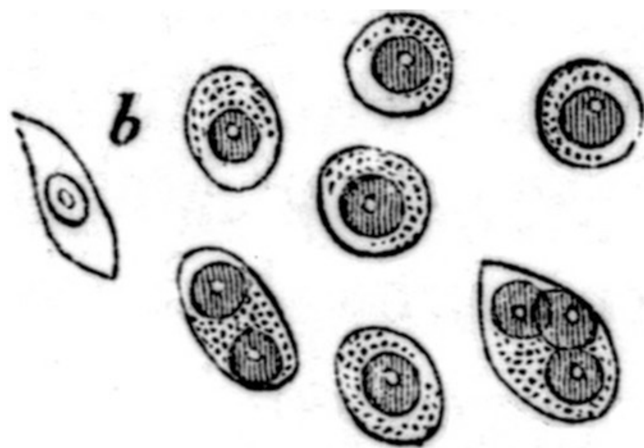

Fig. 24.1 Plasma cells obtained at the autopsy of Thomas Alexander McBean, January 1846 (wood engravings made from drawings by Mr. Dalrymple). https://commons.wikimedia.org/wiki/File:Plasmocytom-dalrymple.PNG; see also Dalrymple [7])

was considered in the differential diagnosis because of the albuminous matter in the urine, but there was no dropsy, and the kidneys appeared healthy. The diarrhea, weakness, emaciation, hepatic enlargement, flatulence, dyspepsia, edema of the ankles, puffiness of the face, and large amounts of Bence Jones proteinuria suggest to contemporary readers the possibility of amyloidosis in addition to myeloma. However, autopsy findings of a normal heart and kidneys and "voluminous liver of healthy structure" make the presence of amyloidosis less likely. Because the lardaceous or waxy changes of amyloidosis in the liver were commonly recognized during the 1840s (although not yet understood), it is unlikely that gross changes of amyloidosis would have been overlooked if present in Mr. McBean [8].

In 1967, Bristol experimental pathologist John Clamp located Mr. McBean's death certificate in the General Register Office in London, using dates and descriptions of the patient from the case reports of Drs. Macintyre and Bence Jones. Dr. Clamp suggested that the name *multiple myeloma* might instead have been *McBean's disease with Macintyre's proteinuria* [3]. However, although Dr. Macintyre first noticed the peculiar heat properties of Mr. McBean's urine, it was Bence Jones who emphasized its place in the diagnosis of myeloma—as he emphasized, "I need hardly remark on the importance of seeking for this oxide of albumen in other cases of mollities ossium"—and who can be credited with developing the first biochemical test for detection of cancer [1, 9]. The modest Dr. Macintyre stated that his "share in this part of the inquiry, it must have been seen, was very humble… I shall be content if I have succeeded in pointing out to future observers, gifted with the requisite qualifications for conducting researches of a higher order, certain definite and distinctive characters by which a peculiar and hitherto unrecorded pathological condition of the urine may be recognised and identified" [4].

Earlier Cases of Multiple Myeloma

Although the first clear description of multiple myeloma did not occur until the 1846–1850 chemical, clinical, and pathological reports of Bence Jones, Macintyre, and Dalrymple described above (all wrote single-authored publications describing the same individual patient), the disease has undoubtedly existed for centuries. It seems likely, for example, that some of the cases of "mollities ossium" reported in the eighteenth and early nineteenth centuries represent patients who had myeloma. However, without detailed microscopic description of plasma cells such as that provided by Mr. Dalrymple in Mr. McBean's case, or recognition of a unique disease-associated protein such as that first detected by Drs. Bence Jones and Macintyre, it is not possible to be certain.

When examining old skeletons, sharply demarcated spheroid skeletal lesions that are "purely lytic"—i.e., lacking gross evidence of sclerosis or formation of new bone—are suggestive of multiple myeloma, especially when such lesions are multiple and occur in the proximal long bones and axial skeleton [10]. Two male human skeletons with this bony lesion pattern, with estimated ages at death of between 40 and 60 years and dating from 3200 to 500 BCE, were identified from among 905 individuals excavated at Thebes-West and Abydos in Upper Egypt [11], while two similarly affected skeletons were found among 2547 individuals entombed in a rural South German ossuary between 1400 and 1800 AD [12].

Paleopathologists have identified additional ancient bones with features suggestive of multiple myeloma, such as the skeleton of a middle-aged Icelandic female from the eleventh to fifteenth century AD [13], two calvaria from medieval Britain [14], four American Indian skeletons from 200 to 1300 AD [15], and 14 pre-Columbian American skeletons dating back to 3300 BC [16]. The Hunterian Museum of the Royal College of Surgeons in London has in its collection the bones of an approximately 45-year-old Roman soldier with myeloma-like lesions. Suspicious rounded lytic lesions can also be seen in the remains of George Grenville (1712–1770), the Whig Prime Minister whose administration passed the notorious Stamp Act of 1765 that first alienated American colonists from England, and who was autopsied by pioneering Scottish surgeon John Hunter in 1770 [17].

Multiple myeloma with Bence Jones proteinuria occurs spontaneously in contemporary animals [18], raising the possibility that myelomatous lesions might be identifiable in prehistoric nonhuman fossils. Paleontologists have observed multiple lytic defects without evidence of bony remodeling in a few dinosaur skeletons from the Jurassic and Cretaceous periods, and these have been interpreted by some observers as evidence of an origin of multiple

myeloma in the Mesozoic era or earlier [19]. However, caution is indicated in interpreting such ancient specimens.

In any case, it is almost certain that 39-year-old Sarah Newbury, a patient described by distinguished London surgeon Samuel Solly in 1844, had multiple myeloma [20]. Mrs. Newbury had experienced increasing fatigue and, 4 years before her death, was suddenly seized with a violent pain in her back when stooping. "Rheumatic pains" in her limbs occurred a year later, and these progressed to the point where her gait became unsteady. Her limb pain increased greatly after a fall in February 1842, which resulted in inability to lift her right leg, and she was confined to her room. Two months later, her femurs fractured "into a thousand pieces" when her husband, a policeman, lifted her from the fireside to carry her to her bed. Pathological fractures of the clavicles, right humerus, and right radius and ulna followed.

Mrs. Newbury first saw Solly in consultation in October 1843, but thereafter wrote him a letter asking him not to visit her again because she was vexed that he had prescribed a bitter infusion to try to improve her poor appetite. She was then lost to medical follow-up until she was admitted at the insistence of her husband to St. Thomas's Hospital in Southwark (South London) on April 15, 1844, in an extremely debilitated state. Over the next few days she was treated with an infusion of orange peel and a rhubarb pill, fed with bland foods including arrowroot, and given an opiate at night if required. Examination of the copious amount of urine she produced revealed a "large quantity of phosphate of lime," but blood tests could not be performed because Mrs. Newbury was "too suspicious and irritable" to allow them [20]. She died suddenly on April 20, 1844, of "asphyxia" [20].

At autopsy, Mrs. Newbury's thoracic cavity was reduced to just 4 in. in transverse diameter. The right lung was compressed to about one-fourth of its natural size, and the left lung was decreased to one-half the extent of the right lung because of skeletal changes. The cancellous portion of the sternum (Fig. 24.2) had been replaced by a red substance similar to that seen in Mr. McBean [4]. A peculiar red material had also replaced much of the femur; it ranged in color "from a deep Modena red to a bright scarlet crimson" (Fig. 24.3). Solly examined this red matter with John Birkett of Guy's Hospital, a surgeon and anatomist, who described the cells within as "very clear, their edge being remarkably distinct, and the clear *oval* outline enclosing *one* bright *central* nucleus, *rarely two, never more*" [italics Solly's] [20]. John Dalrymple noted that the microscopic appearance reported by Birkett "accords very nearly" with his description of Mr. McBean's marrow [7]. Solly postulated that the process affecting Mrs. Newbury was inflammatory and had begun with an abnormality of the blood vessels, in which the "earthy matter of the bone is absorbed and thrown out by the kidneys in the urine" [20].

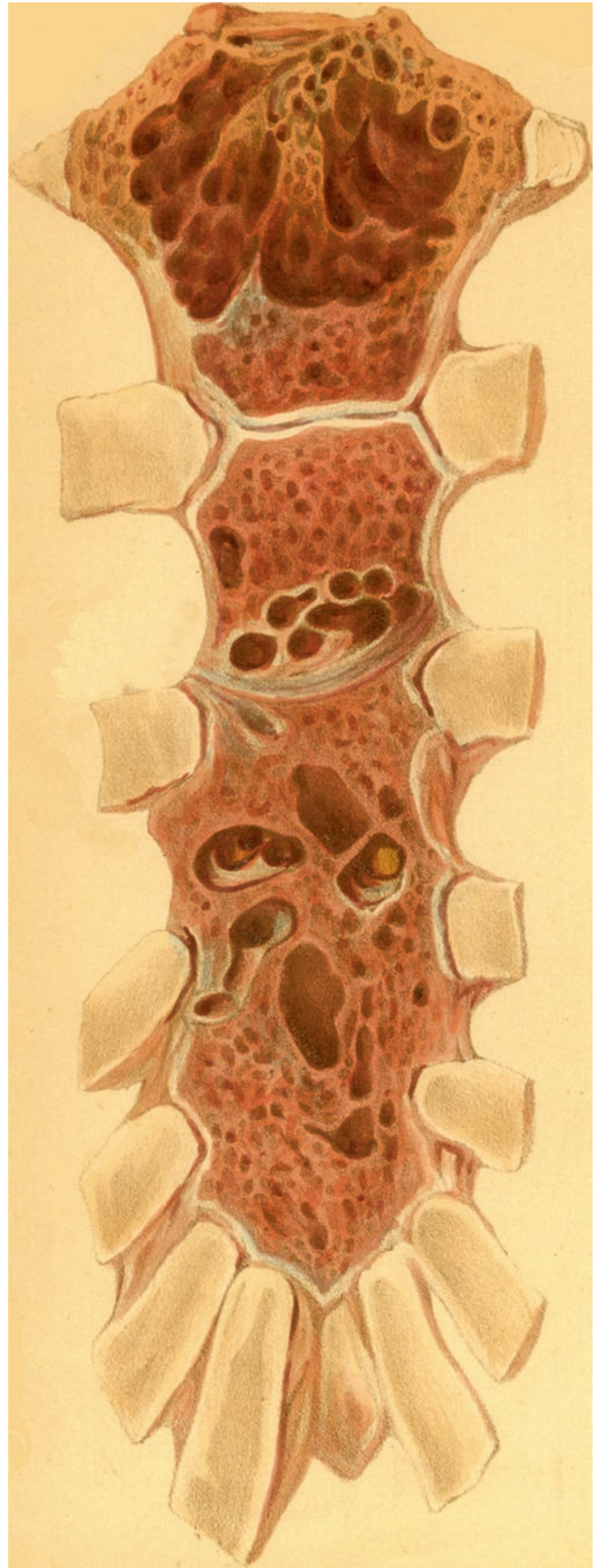

Fig. 24.2 Sternum of Sarah Newbury showing destruction of bone; drawn after her autopsy in April 1844 (from Solly [20])

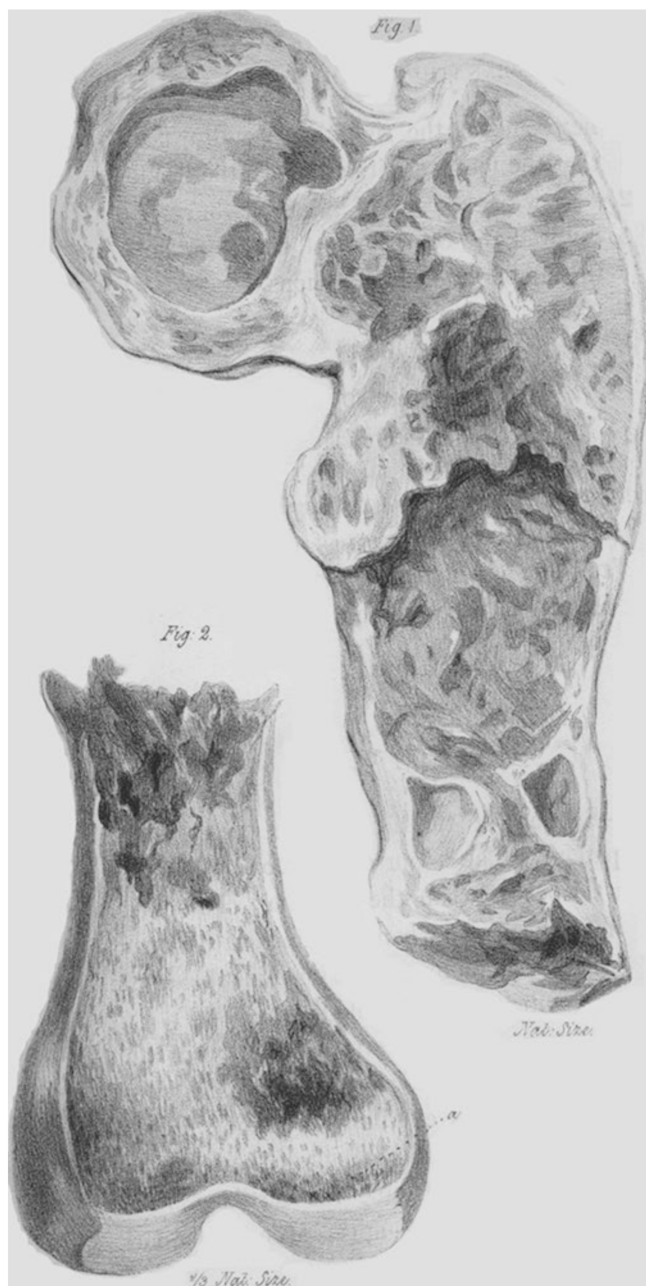

Fig. 24.3 Femur of Sarah Newbury showing destruction by myeloma tumor (from Solly [20])

Other Contributions to Bence Jones Proteinuria and Monoclonal Protein Detection

Although much has been written about the life and scientific work of Henry Bence Jones [2, 21–23], a number of other persons played a part in the evolving story of Bence Jones proteinuria.

In 1846, Johann Florian Heller, an Austrian chemist and physician working in Vienna, described a protein in the urine of a patient that precipitated when warmed a little above 50 °C and disappeared upon further heating [24]. Although Heller did not recognize the re-precipitation of the protein when the urine was cooled, it seems likely that he was observing a Bence Jones protein. Heller also distinguished this new protein from albumin and casein [24]. Richard Fleischer in Erlangen, Germany, was the first to use the term "Bence Jones protein" in writing, in an 1880 paper describing a substance with chemical characteristics of Bence Jones protein that he isolated from normal bone marrow [25].

In 1883, Wilhelm Friedrich Kühne, a prominent Berlin physiologist best known for coining the term "enzyme," described finding Bence Jones protein in the urine of a 40-year-old patient from Amsterdam who died in 1869 after an illness characterized by bone pain, spinal curvature, and cranial neuropathy, possibly from an extramedullary plasmacytoma [26]. Kühne isolated the protein and found that the carbon, hydrogen, and nitrogen levels were similar to those described by Bence Jones, and attributed any differences in results between his report and that of Bence Jones to the fact that his preparation was more pure than Bence Jones's preparation. He named the peculiar protein "albumosurie."

In 1898, Thomas R. Bradshaw of Liverpool observed that meals had little or no influence on the amount of Bence Jones proteinuria [27]. There was no nocturnal variation, and Bradshaw believed that the rate of protein excretion was "pretty constant throughout the 24 hours."

In 1899, Alexander Ellinger in Germany suggested that there might be an abnormal protein in the blood in patients with myeloma that was similar to the Bence Jones protein, but he was unable to prove this assertion [28].

Waltman Walters at Mayo Clinic in Minnesota described three carefully evaluated patients with multiple myeloma in 1921, and, like Bradshaw, reported that the quantity of Bence Jones proteinuria in these patients was independent of their oral protein intake and did not vary diurnally [29]. In one patient, intravenous injection of Bence Jones protein appeared to increase the amount of Bence Jones proteinuria. Walters also found Bence Jones protein in the blood of one patient and in the bronchial secretions of another. He concluded that Bence Jones proteins were of endogenous origin, and hypothesized that they were derived from blood proteins through some type of action of abnormal cells in the bone marrow [29].

The following year—1922—Stanhope Bayne-Jones and D.W. Wilson from Johns Hopkins made 12 preparations of Bence Jones proteins from five patients, two of whom had been included in Walters' report [30]. Bayne-Jones and Wilson immunized rabbits by intravenous injection of the Bence Jones protein, and performed precipitin tests with the Bence Jones protein preparations, concluding that Bence Jones proteins consisted of two groups of similar but not identical proteins.

Beginning in the 1930s, there was considerable debate between proponents of two schools of thought on the origin of Bence Jones protein. The first theory, championed by Adolf Magnus-Levy in Berlin, held that proteinaceous materials found in the urine were the result of overproduction of normal serum proteins by the bone marrow [31]. An alternative view was held by Maxwell Wintrobe and Mar Van Rensselaer Buell at Johns Hopkins in Baltimore. In a description of the phenomenon of cryoprecipitation in 1933, Wintrobe and Buell argued that pathologic proteins in plasma cell disorders were likely to be distinct from all normal serum components [32].

In the early 1950s, protein chemist Frank W. Putnam at the University of Chicago performed a series of experiments in myeloma patients using ^{13}C radioisotopes, which helped clarify the origin of Bence Jones proteins. Putnam first showed that the Bence Jones proteins from 18 different patients with myeloma were each biochemically unique, though as had been suggested by Bayne-Jones and Wilson they clustered into two antigenic groups. In 1955, Putnam and his coworker Sarah Hardy showed that Bence Jones proteins derived directly from the body's metabolic pool of nitrogen, rather than being a breakdown product of some sort of plasma precursor [33].

In 1956, Leonhard Korngold and Rose Lipari at New York's Sloan Kettering Institute for Cancer Research and Cornell Medical College Department of Biochemistry formally demonstrated a relationship between Bence Jones protein and the serum proteins of multiple myeloma [34]. As a tribute to Korngold and Lipari, the two major classes of Bence Jones proteins are designated by the Greek letters κ and λ.

One hundred and seventeen years after the initial description of the unique heat properties of Bence Jones protein—in 1962—Gerald Edelman and Joseph Gally at the Rockefeller Institute for Medical Research in New York demonstrated that the light chains prepared from a serum immunoglobulin G (IgG) myeloma protein and the Bence Jones protein from the same patient's urine were identical in all respects: the same amino acid sequence, similar spectrofluorometric behavior, the same molecular weight, identical appearance on chromatography with carboxymethylcellulose and on starch gel electrophoresis after reduction and alkylation, and the same ultracentrifugal pattern—as well as the same thermal solubility [35]. The light chains precipitated when heated to between 40 and 60 °C, dissolved on boiling, and reprecipitated when cooled to between 40 and 60 °C.

Shortly after Edelman and Gally's discovery, Norbert Hilschmann and Lyman Craig of the Rockefeller Institute [36] and Koiti Titani and colleagues in Putnam's laboratory [37] provided the first antibody amino acid sequences, and showed that Bence Jones proteins were not only related to the light chains of gamma globulin but also that each light chain was divided into a "variable" or V region, and a "constant" or C region. This structure accounts for the heterogeneity of normal gamma globulins and for antibody specificity and diversity.

Diagnostic Tests for Myeloma: Beyond Bence Jones Proteinuria

Arne Tiselius in Uppsala, Sweden, a 1948 Nobel Laureate in Chemistry, reported an improved method of serum electrophoresis in 1937, which allowed separation of serum globulins into three components: alpha, beta, and gamma [38]. In 1939, Tiselius isolated antibody activity to the gamma fraction [39], while Lewis Longsworth and colleagues at the Rockefeller Institute first noted the classic myeloma "M-spike" in that same year, using Tiselius' electrophoretic techniques [40].

In 1953, Pierre Grabar and Curtis A. Williams at the Institut Pasteur in Paris described immunoelectrophoresis, a technique that is infrequently practiced today but routinely facilitated the diagnosis of multiple myeloma [41]. Immunofixation or "direct immunoelectrophoresis" was reported by Armine T. Wilson from the Alfred I. duPont Institute in Wilmington, Delaware, in 1964 and is now the standard method for the recognition of monoclonal proteins [42]. Wilson applied antisera on the surface of the agar immediately after completion of electrophoresis. Immunofixation has proven useful when the results of immunoelectrophoresis are equivocal [43], and is also helpful in the recognition of small monoclonal light chains not detectable by immunoelectrophoresis [44].

Nonsecretory multiple myeloma is defined by the absence of detectable monoclonal proteins in serum and urine using immunoelectrophoresis or immunofixation; it accounts for <3% of patients with multiple myeloma. In 2001, Arthur J. Bradwell from Birmingham, England, and his colleagues reported an immunological method for detecting imbalance in the concentration of κ and λ serum free light chains, using specific antibodies that bind only to free light chains, not to light chains bound to immunoglobulin heavy chains [45]. Serum free light chain concentrations are now routinely measured in clinical practice.

Early Cases of Multiple Myeloma After Sarah Newbury and Thomas McBean

In 1867, Hermann Weber, a German physician working in London, described a 40-year-old man with mollities ossium and first linked this condition to the pathological finding of tissue deposition of amyloid [46]. The patient suffered severe sternal and lumbar pain, and movement of his head produced pain in his neck and arms. The patient died less than 4 months

after the initial onset of pain. Postmortem examination revealed that the sternum was fractured in two places, and had been almost entirely replaced by a grayish-red substance that was thought to have the microscopic appearance of a sarcoma. Several round defects in the skull were also filled with the same morbid substance as that found in the sternum, and many of the ribs, several vertebrae, and parts of the pelvis were involved by the same process. The waxy changes of amyloid were found in the kidneys and spleen.

During a meeting of the Pathological Society of London in February 1872, William Adams exhibited (on behalf of Thomas Stretch Dowse of Highgate Infirmary) specimens from the body of a 62-year-old woman with "acute rheumatism" characterized by bone pain, fractures, and fever, who died 8 days after admission to the hospital for a humerus fracture. The left femur fractured while the body was being placed on the autopsy table. Lardaceous changes consistent with amyloid were found in the liver and kidneys, and the cancellous portions of the bones had been replaced by a homogeneous, soft, gelatinoid substance. When examined microscopically, the substance filling the hollowed bone was shown to consist of small spherical and oval cells that contained one eccentric oval nucleus (rarely two) [47].

The term "multiple myeloma" was introduced in 1873 by J. von Rusitzky from Kiev, of whom little is known other than that he had once worked in Friedrich von Recklinghausen's laboratory in Strasbourg. During an autopsy, von Rusitzky noted eight separate tumors of the bone marrow in a patient, which he called "multiple myelomas" [48]. The patient, a 47-year-old man, had presented with a gradually enlarging tumor in the right temple. Subsequently, thickening of the sternal manubrium and the seventh rib developed, followed by paraplegia. At autopsy, it was revealed that a fist-sized tumor in the right frontal region extended into the orbit and had produced ophthalmoplegia. Other postmortem findings included an apple-sized tumor in the right fifth rib, a tumor in the left seventh rib that produced a fracture, a tumor of the sternum, a tumor involving the sixth to the eighth thoracic vertebrae (the cause of the paraplegia), and three tumors of the right humerus. Although von Rusitzky's description of the tumor cells in this case is vague, he described round cells with a nucleus located in the periphery near the cell membrane, suggestive of plasma cells. The report did not comment on whether Bence Jones proteinuria was found during life. In Russia and Soviet-influenced regions, myeloma was called "von Rusitzky syndrome" for much of the twentieth century.

There were few further publications about the disease until 1889, when Otto Kahler, an internist from Prague working in Vienna, described the case of a 46-year-old physician named Dr. Loos [49]. In July 1879, Dr. Loos developed sudden severe pain in the right upper thorax, which was aggravated by taking a deep breath. Six months later, this pain recurred and became localized to the right third rib, which was tender to pressure. During the next 2 years, intermittent pain aggravated by exercise occurred in the ribs, spinal column, left shoulder, upper arm, and right clavicle. Albuminuria was first noticed in September 1881. Skeletal pain, made worse by movement, continued to occur intermittently. Pallor was noted in 1883 and pneumonia developed in February 1884. In December 1885, Dr. Loos was first seen by Kahler, who noted anemia and focal tenderness of many bones. Kyphosis was so severe that when Dr. Loos stood up, his lower ribs touched his anterior iliac crest. Dr. Loos subsequently suffered from recurrent bronchial infections and intermittent hemoptysis. During the following year, Dr. Loos' kyphosis increased and height decreased monthly, to the point where his chin pressed against the sternum, resulting in skin ulceration. On August 26, 1887, Dr. Loos died, a remarkable 8 years after his initial symptoms.

Kahler's autopsy report of Dr. Loos described hepatosplenomegaly, but lardaceous change was not mentioned. The ribs were soft and could be broken with minimal effort. Soft gray-reddish masses were noted in the ribs and thoracic vertebrae. Microscopic examination showed large round cells, consistent with myeloma. It is interesting to note that the patient had a high fluid intake and took sodium bicarbonate on a regular basis; this regimen may have helped prevent renal failure. Kahler recognized that the urinary protein obtained from Dr. Loos had the same characteristics that Bence Jones had described. For many years, the eponym "Kahler's disease" was widely used in Western Europe and the United States to describe the condition once known by "mollities ossium with Bence Jones proteinuria" and now called multiple myeloma.

Detailed examination of Dr. Loos' urine by Karl Hugo Huppert, professor of medicinal chemistry at the German University in Prague, showed that a protein in the urine precipitated at 53–59 °C, cleared with heating to boiling, and then reprecipitated during cooling [50]. The patient excreted 6.7 g of the protein daily, which Huppert noted was distinct from albumin.

It is likely that A.L., the 43-year-old engineer that Joseph Coats of Glasgow reported in 1891 as having "multiple sarcoma of bone," actually had multiple myeloma [51]. The patient developed a large tumor of the sternum 5 years before his final hospitalization and death, and later noticed tumors in the right clavicle, right humerus, and left hip. He experienced back pain radiating to the lower extremities, weakness of his legs, and a pathological fracture of the right humerus. Postmortem examination revealed multiple tumors with involvement of the ribs and vertebral bodies. Microscopic examination showed round or polygonal cells "about 1/2000 in. diameter" (~13 μm, a reasonably accurate estimate of the diameter of a neoplastic plasma cell) with oval nuclei constituting more than half the diameter of the cells [51].

An 1897 Italian description of Kahler's disease by Camillo Bozzolo, a Milanese physician and pathologist working in Torino, resulted in the dual eponym "*malattia di Kahler-Bozzolo"* gaining currency in Italy in the first part of the twentieth century [52].

Other Cases of Multiple Myeloma in the Nineteenth and Twentieth Centuries

In 1894, James Bryan Herrick and Ludvig Hektoen at Rush Medical College in Chicago reported what is probably the first recognized case of multiple myeloma in the United States, just a few years after the report of Kahler [53]. A 40-year-old woman complained of lumbar pain and a nodule on the lower end of the sternum. At autopsy, there were multiple nodules attached to the sternum, right clavicle, and ribs. The sternum was thickened, irregular, and covered with tumor masses, yet was soft and flexible. Multiple nodules were found on the ribs, which bent readily without cracking. Two of the dorsal vertebral bodies were largely replaced by soft tumor masses, and fungoid masses were seen in the skull. Microscopic examination of these lesions revealed round "lymphoid" cells with large nuclei. Herrick is also credited with the first clear description of sickle cell disease (initially recognized by one of his subordinates), as well as being the first to link angina with acute coronary syndromes [54, 55].

In 1898, Frederick Parkes Weber—honorary physician to the German Hospital in Queen's Square, London, and son of Hermann Weber mentioned above—reported a case of multiple myeloma, and suggested that radiographs (discovered by Würzburg physicist Wilhelm Conrad Röntgen in 1895) would greatly facilitate the diagnosis of such cases [56]. Weber also concluded in a report of another case that Bence Jones protein was produced by the bone marrow, and that the presence of Bence Jones protein was of "fatal significance" and nearly always indicated that the patient had multiple myeloma [57]. Weber and Lister Institute bacteriologist John Charles Grant Ledingham later suggested that Bence Jones protein derived from cytoplasmic residua of karyolyzed plasma cells [58].

In 1928, C.F. Geschickter and M.M. Copeland from Johns Hopkins reviewed all 425 cases of multiple myeloma reported since 1848 [59]. They called attention to six cardinal features of the disease: multiple tumors of the axial skeleton, pathological fractures, Bence Jones proteinuria, back pain, anemia, and chronic renal disease.

Sternal aspiration of bone marrow during life, described by Soviet physician Mikhail Arinkin in Leningrad in the late 1920s, greatly increased the recognition of multiple myeloma [60]. In 1938, hematologists Nathan Rosenthal and Peter Vogel at Mt. Sinai Hospital in New York reported that only 3 cases of multiple myeloma had been recognized at the Mt. Sinai Hospital from 1916 to 1935, but that 13 cases were found in the ensuing 2 1/2 years [61]. Rosenthal and Vogel attributed this marked increase in recognition to the use of sternal puncture in patients with obscure anemia or skeletal abnormalities, and suggested that many cases of myeloma had likely been missed in the past.

In 1947, Edwin (Ned) Bayrd and Frank Heck at Mayo Clinic in Minnesota described 83 patients with histological proof of myeloma seen at their institution through December 1945 [62]. The duration of survival in the Mayo series ranged from 1 to 84 months (median, 15 months). The median survival for myeloma would not increase much beyond this until the introduction of novel agents in the early twenty-first century [63].

Plasma Cells, Hyperproteinemia, Recognition of Monoclonality, and Prognosis

The term *plasma cell* was coined by German anatomist Heinrich Wilhelm Gottfried von Waldeyer-Hartz in 1875, but his description is not characteristic of plasma cells, and it is most likely that he was instead observing tissue mast cells [64]. Plasma cells were described accurately by Spanish neuroscientist Santiago Ramón y Cajal in 1890, during the study of syphilitic condylomas. Ramón y Cajal stated that the unstained perinuclear area ("hof," from a German term meaning yard or court) contained the Golgi apparatus. In 1891, German dermatologist Paul Gerson Unna used the term *plasma cell* while describing cells seen in the skin of patients with lupus erythematosus [65]. However, it is not known whether he actually saw plasma cells. In 1895, Hungarian pathologist Tamás Marschalkó outlined the essential characteristics of plasma cells, including blocked chromatin, eccentric position of the nucleus, a perinuclear pale area ("hof"), and a spherical or irregular cytoplasm [66].

In 1900, pathologist J.H. Wright of Johns Hopkins described a 54-year-old man with multiple myeloma, and pointed out that the tumor consisted of plasma cells, proposing the new term "plasma cell myeloma" [67]. Wright emphasized that the neoplasm originated not from red marrow cells collectively but from only one type of cell, the plasma cell. Interestingly, Wright's patient was probably the first in whom radiographs revealed changes in the ribs, thus contributing to the diagnosis.

Although Victor C. Jacobson of the Peter Bent Brigham Hospital in Boston reported Bence Jones protein in the serum in a patient with chronic nephritis in 1917 [68], it was not until 1928 that biochemist William A. Perlzweig and colleagues from Duke University reported hyperproteinemia when they described a patient with multiple myeloma who had 9–11 g of globulin in his serum [69]. The patient also

had Bence Jones proteinuria and probably a small amount of Bence Jones protein in the plasma. Perlzweig and coworkers noted that it was almost impossible to obtain serum from the clotted blood because the clot failed to retract, even on prolonged centrifugation.

The concept of monoclonal versus polyclonal gammopathies was lucidly presented in the 1961 edition of the Harvey Society Lecture series (founded 1905) in New York by Jan Gösta Waldenström of Malmö General Hospital, Sweden [70]. Waldenström clearly described patients with a narrow band of hypergammaglobulinemia on electrophoresis as having a monoclonal protein. Although many of these patients had multiple myeloma, others had no evidence of malignancy and were considered to have idiopathic "essential hypergammaglobulinemia" or benign monoclonal gammopathy. Most physicians now use the term "monoclonal gammopathy of undetermined significance" (MGUS) instead, because in some of these patients, multiple myeloma, macroglobulinemia, or a related disorder will eventually develop [71–73]. Waldenström further correctly regarded the broad band in hypergammaglobulinemia as a polyclonal increase in proteins. This simple distinction is extremely important clinically because patients with a monoclonal gammopathy already have or may develop a neoplastic process, whereas patients with a polyclonal gammopathy have an inflammatory or a reactive cause of their hypergammaglobulinemia [70, 74].

In 1975, Brian G.M. Durie and Sydney Salmon proposed a three-tier myeloma staging system with prognostic value [75]. The Durie-Salmon staging system saw widespread clinical use for more than 30 years but had limitations, particularly with respect to categorization of bone lesions. In 2005, a multinational group chaired by Philip Greipp from Mayo Clinic used data from more than 11,000 patients to develop a simplified International Staging System, based on serum β (beta) 2-microglobulin and albumin levels [76]. Other important prognostic factors in myeloma defined in recent years include specific chromosomal abnormalities detectable by fluorescent in situ hybridization or conventional karyotyping, such as deletion of chromosomes 13 or 17p, abnormalities of chromosome 1, hyperdiploidy, or immunoglobulin heavy-chain translocations such as t(4;14), t(11;14), or t(14;16) [77]. A growing number of somatic mutations have been described in myeloma cells, including mutations in genes important in protein synthesis, histone modulation, coagulation, and NF-kappaB signaling [78].

Treatment of Multiple Myeloma

Treatment of multiple myeloma has progressed considerably since the nonspecific remedies attempted for Mr. McBean in 1845, especially with the introduction of highly active novel agents including immunomodulatory drugs and proteasome inhibitors [63]. Despite these novel drug therapies and the routine use of autologous stem cell transplantation for patients with myeloma [79], which have improved survival in recent years [64], the condition remains incurable.

The Urethane Distraction

In 1947, Nils Alwall, a Swedish dialysis pioneer, reported a patient with multiple myeloma who experienced a reduction in globulin levels from 5.9 to 2.2 g/dL, an increase in hemoglobin, disappearance of proteinuria, and reduction in bone marrow plasma cells from 33 to 0% when treated with urethane (ethyl carbamate) [80]. On the basis of this single report, for almost 20 years urethane was commonly used as a treatment of myeloma. The demise of urethane in myeloma therapy occurred in 1966, when James Holland and his colleagues reported results from a randomized trial of 83 patients with previously treated or untreated multiple myeloma who received either urethane or a placebo consisting of cherry- and cola-flavored syrup [81]. No difference was seen in objective improvement between the two treatment groups, and the urethane-treated patients died earlier on the average than those treated with placebo. This difference was ascribed to urethane-induced azotemia.

Alkylating Agents and Combination Chemotherapy

In 1958, a Soviet medical team led by Nikolai Blokhin reported benefit in three of six patients with multiple myeloma who were treated with "sarcolysin" (L-phenylalanine mustard, melphalan, Alkeran) [82]. Four years later, Daniel Bergsagel at the M.D. Anderson Cancer Center in Houston, Texas, and his colleagues observed improvement in 8 of 24 patients with multiple myeloma who were treated with melphalan [83].

In a 1964 report, cyclophosphamide-treated patients with myeloma had a median survival of 24.5 months, whereas a control myeloma group had a median survival of 9.5 months [84]. Objective improvement occurred in 81 of 207 cyclophosphamide-treated patients. Various combinations of alkylating agents with vinca alkaloids, anthracyclines, and corticosteroids (see below) were used for the treatment of multiple myeloma, but these combinations did not prove superior to melphalan and prednisone [85, 86].

Corticosteroids

Corticosteroids were first systematically tested in patients with myeloma by R.E. Mass in 1962, who reported the

results of a placebo-controlled double-blind trial in which single-agent prednisone decreased serum globulin and increased hematocrit, but did not improve survival [87]. In 1967, Sydney Salmon and his colleagues reported that prednisone administered at a dose of 200 mg orally every other morning produced benefit in 8 of 10 patients with poor-risk myeloma, and adverse events were uncommon [88]. In two Cancer and Leukemia Group B myeloma treatment protocols, prednisone as a single agent produced a 44% objective response rate [89]. The combination of melphalan plus prednisone (MP) was established in a randomized trial of 183 myeloma patients led by Raymond Alexanian and colleagues; patients treated with MP lived 6 months longer than those who received melphalan alone [90]. Later dexamethasone became the corticosteroid of choice in myeloma therapy.

Stem Cell Transplantation

The first report of human hematopoietic stem cell transplantation was published in 1957 by E. Donnall Thomas and Joseph Ferrebee at the Mary Imogene Bassett Hospital in Cooperstown, New York, and their colleagues [91]. Thomas and Ferrebee treated six patients (one had multiple myeloma) with total-body irradiation and chemotherapy followed by an intravenous infusion of bone marrow cells from a healthy donor. Although none of the patients lived 100 days, and transient engraftment occurred in only two of the six patients, Thomas remained convinced of the procedure's potential and continued to purse it, in collaboration with immunologists who developed the concept of tissue (human leukocyte antigen) typing [92]. Thomas moved to Seattle in 1963, and his groundbreaking research on stem cell transplantation eventually led to a Nobel Prize in 1990.

The Seattle group reported a syngeneic bone marrow transplant in a patient with multiple myeloma in 1982; the patient's physician brother served as donor [93]. Four years later, Alexander Fefer from Seattle and his colleagues described five myeloma patients who received a syngeneic bone marrow transplant [94]. In 1987, a European group led by Gösta Gahrton of Sweden reported that 10 of 14 patients with multiple myeloma who received an allogeneic bone marrow transplant from an HLA-compatible sibling donor survived for at least 6 months (median, 12 months) [95].

The first autologous bone marrow transplantation for myeloma was reported in 1983 by Tim McElwain and Ray Powles from the Institute of Cancer Research and the Royal Marsden Hospital in the United Kingdom [96]. Four years later, Bart Barlogie and his colleagues at the University of Arkansas reported the use of melphalan 140 mg/m^2 and total-body irradiation (1.5 Gy) followed by autologous allogeneic bone marrow transplantation in multiple myeloma patients refractory to chemotherapy [97]. Subsequently, Barlogie developed intense treatment programs incorporating tandem autologous transplantation, which he called "total therapy"—a term and concept pioneered for childhood leukemia at St. Jude Children's Research Hospital in Memphis. Subsequent clinical trials of transplantation regimens played a major role in establishing high-dose therapy and stem cell rescue as a standard treatment for multiple myeloma.

Novel Agents

Since the late 1990s, several exciting new agents have emerged and achieved regulatory approval, which has improved outcomes for patients with myeloma [63].

Thalidomide, Lenalidomide, and "Immunomodulatory" Agents

The tragedy of thalidomide embryopathy is widely known outside of the medical profession, but the drug's rebirth as an immunomodulatory and antineoplastic agent is less well recognized.

In 1957, German pharmaceutical company Chemie Grünenthal began marketing thalidomide (α-N-[phthalimido] glutarimide) as a sedative. By 1960, thalidomide was sold in more than 40 countries, and achieved widespread use as a sedative that was thought to be safer than barbiturates, as well as a treatment for morning sickness of pregnancy [98].

On November 18, 1961, Widukind Lenz, a German pediatrician and geneticist, reported that thalidomide exposure during the first trimester of fetal development was associated with severe teratogenic malformations [99]. In December 1961, Lenz' findings were independently confirmed by an Australian obstetrician, William G. McBride [100]. By the end of 1961, thalidomide was removed from the market in most countries, but almost 10,000 infants had already been affected. The United States was largely spared because thalidomide had been denied approval by Frances Oldham Kelsey at the US Food and Drug Administration (FDA), due to a lack of safety data.

Thalidomide persisted as a therapeutic agent due to promising activity later seen in leprosy (1964), Behçet disease (1979), graft-versus-host disease (1988), and human immunodeficiency virus (HIV)-associated oral ulcers and wasting (1989) [98]. Under pressure from activists, the FDA approved thalidomide for the treatment of erythema nodosum leprosum in July 1998, with a risk-management system to control prescribing and prevent exposure of unborn infants to thalidomide.

In 1994, the antiangiogenic properties of thalidomide in a rabbit cornea micropocket assay were reported [101].

Based on the increasing awareness of angiogenesis in the pathobiology of cancer and the evidence of increased angiogenesis in myeloma, the spouse of an affected myeloma patient convinced Barlogie and his colleagues to initiate a compassionate-use trial of "antiangiogenic therapy" at the University of Arkansas in late 1997. The idea to use thalidomide in this setting came from Harvard's Judah Folkman, an angiogenesis researcher [102]. In an Arkansas study of 84 previously treated patients, 32% responded to thalidomide. Other investigators subsequently confirmed the Arkansas results and observed activity of thalidomide in newly diagnosed myeloma as well [103]. Although it was never clear whether the mechanism of action required inhibition of angiogenesis—various drug-associated changes in cytokines and immune cell subsets led to thalidomide and its derivatives also being called an "immunomodulatory agent" (iMid)—thalidomide soon became a therapy widely used in myeloma, including in combination with corticosteroids and other agents.

The sponsor of thalidomide subsequently synthesized several analogues to try to increase clinical activity and minimize adverse events such as neuropathy. The most successful of these analogs has been lenalidomide (formerly CC-5013), a 4-amino-substituted analog of thalidomide. A Phase 1 trial in 24 relapsed refractory myeloma patients was led by Paul G. Richardson at the Dana-Farber Cancer Institute; as reported in 2002, lenalidomide reduced paraprotein levels by at least 25% in 17 of 24 patients [104].

Richardson and colleagues then conducted a multicenter randomized Phase 2 trial that enrolled 102 patients with relapsed/refractory myeloma and confirmed the drug's activity [105]. In a Mayo Clinic study of lenalidomide and dexamethasone of 34 patients with newly diagnosed myeloma, 91% achieved an objective response with lenalidomide plus dexamethasone [106]. Lenalidomide plus dexamethasone was approved by the FDA in June 2006 for the treatment of myeloma in patients who failed one prior therapy; lenalidomide had previously been approved in 2005 for treatment of lower risk myelodysplastic syndromes associated with deletions of chromosome 5q. Numerous studies showed benefit of lenalidomide in combination regimens both as initial therapy and in relapsed/refractory disease. Lenalidomide was soon widely used in maintenance regimens for at least 2 years after stem cell transplant, which prolonged relapse-free survival in several studies [107]. Another immunomodulatory agent/thalidomide analogue, pomalidomide, received accelerated approval for patients with relapsed/refractory disease in 2013 [108].

The mechanism of thalidomide and lenalidomide in myeloma was only discovered in 2014, after both drugs had been in use for many years [109]. These drugs were found to bind to cereblon, a component of an E3 ubiquitin ligase complex, altering the affinity of this complex for various proteins and thereby changing their rate of degradation. In myeloma cells, increased ubiquitin ligase-mediated degradation of the transcription factors IKZF1 and IKZF3 results in cytotoxicity, since these factors are necessary for malignant plasma cell survival.

Proteasome Inhibitors

The ubiquitin-proteasome pathway contributes to the orderly degradation of unneeded eukaryotic cellular proteins [110]. Inhibition of the proteasome leads to cellular apoptosis, especially in neoplastic and rapidly proliferating cells [111].

Bortezomib (formerly PS-341), a derivative of boronic acid, was synthesized in 1995 and selected for preclinical and clinical testing in cancer [111]. In a Phase 1 study enrolling patients with relapsed and refractory hematological malignancies led by Robert Orlowski at the University of North Carolina and published in 2002, bortezomib demonstrated striking antimyeloma activity [112]. Kenneth Anderson at the Dana-Farber Cancer Institute in Boston and his colleagues also observed promising activity of bortezomib against myeloma cells in several preclinical models [113].

In the first Phase 2 trial of bortezomib in 202 patients with relapsed refractory myeloma, approximately one-third of patients responded to bortezomib therapy, with a median response duration of 1 year [114]. These results led the FDA to approve bortezomib for multiple myeloma in May 2003. Bortezomib has also been combined effectively with corticosteroids and with lenalidomide, and used in newly diagnosed patients. Subsequently several other proteasome inhibitors were developed, including carfilzomib (FDA approved for relapsed/refractory myeloma in mid-2012) and ixazomib (FDA approved in late 2015) [115, 116]. In 2015, in a cooperative group trial, a combination regimen of bortezomib, lenalidomide, and dexamethasone (VRd) was shown to improve overall survival compared with lenalidomide and dexamethasone (Rd) alone—the first time a "triplet" combination had shown benefit compared to a "doublet" [117].

Monoclonal Antibodies and Other Agents

Following the success of the anti-CD20 antibody rituximab in non-Hodgkin lymphoma, numerous monoclonal antibodies were developed for other hematological neoplasms. Antibodies used in myeloma include elotuzumab, a humanized antibody targeting signaling lymphocytic activation molecule F7 (SLAMF7), and daratumumab, a fully human antibody against CD38, which were both FDA approved in late 2015 [118, 119]. Panobinostat, a deacetylase inhibitor, also has activity in relapsed/refractory myeloma and was FDA approved in early 2015 [120]. Several other novel

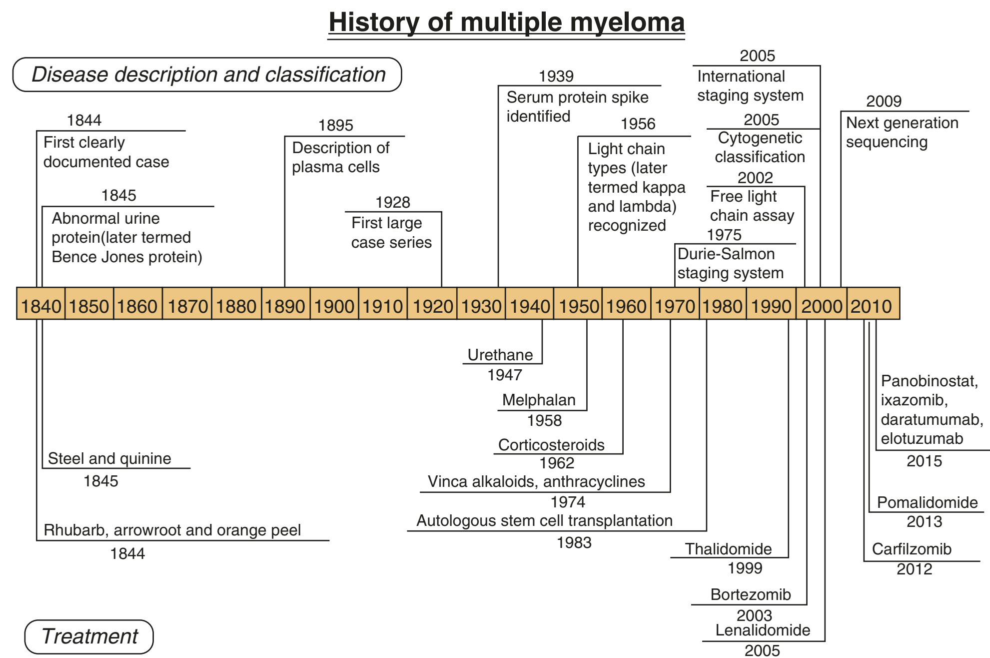

Fig. 24.4 Timeline of historical discoveries (*top*) and introduction of specific therapies (*bottom*) for multiple myeloma. For therapies approved in the twenty-first century, the date of initial FDA approval is given, rather than the first date these agents were used in humans (revised and updated from Kyle and Rajkumar [121])

agents are in various stages of development. After many years of limited therapeutic alternatives, the future looks brighter for patients with myeloma (Fig. 24.4).

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25 Monoclonal Gammopathy of Undetermined Significance

Malin Hultcrantz and Ola Landgren

Introduction

The presence of a monoclonal protein on serum electrophoresis in otherwise healthy individuals was first described by Prof. Jan Waldenström who named this condition "essential hypergammaglobulinemia" in 1960 [1]. Dr. Kyle et al. later observed that individuals with a monoclonal gammopathy were at a higher risk of developing multiple myeloma and therefore coined the term "monoclonal gammopathy of undermined significance" (MGUS) [2]. The diagnostic criteria for MGUS are presence of monoclonal protein <3.0 g/dL, <10% clonal plasma cells in the bone marrow, and absence of end-organ damage that can be attributed to a plasma cell disorder [3]. MGUS can be further classified into non-IgM MGUS, IgM MGUS, and light-chain MGUS (LC-MGUS) [3]. Typically, patients with non-IgM MGUS, IgM MGUS, and LC-MGUS progress to multiple myeloma, Waldenström's macroglobulinemia, and light-chain multiple myeloma, respectively. In addition, MGUS has been associated with comorbidities that are not necessarily caused by the plasma cell dyscrasia [4]. Several host factors, e.g., gender and ethnicity, as well as external factors, e.g., exposure to pesticides, are associated with an increased risk of MGUS [5, 6]. The rate of progression to multiple myeloma is approximately 0.5–1% per year and there is evidence that multiple myeloma is consistently preceded by MGUS [7]. A number of predictive factors as well as prognostic scoring systems have been developed to estimate the risk of progression to malignant disease [7–9]. In this review chapter, we discuss the diagnosis, risk of progression, as well as clinical implications for patients with MGUS.

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Diagnosis and Definition of MGUS

In the majority of patients, MGUS is diagnosed incidentally during workup for other various symptoms or disorders. The diagnostic criteria for MGUS, smoldering multiple myeloma, and multiple myeloma were updated in 2014 [3]. MGUS is defined as presence of a monoclonal protein (M-protein) <3.0 g/dL, <10% plasma cells in the bone marrow, and absence of events defining for myeloma or other plasma cell disorders; all three criteria must be met [3]. Myeloma-defining events are presence of end-organ damage that can be attributed to the plasma cell proliferation as defined by the CRAB criteria (hypercalcemia, renal failure, anemia, bone lesions, Table 25.1) or the recently added definitions of free light chain (FLC) ratio ≥100, >60% clonal plasma cells in the bone marrow, or >1 lytic bone lesion on whole-body MRI [3].

There are three different types of MGUS: *non-IgM MGUS, IgM MGUS,* and *light-chain MGUS* (Table 25.2). Non-IgM MGUS is the most common type and is diagnosed on serum electrophoresis. In non-IgM MGUS, most patients have M-proteins consisting of IgG or IgA while IgD is rare and there are only a few cases in the literature of IgE

Table 25.1 Definition of monoclonal gammopathy of undetermined significance (MGUS), smoldering multiple myeloma (SMM), and multiple myeloma (MM)

	MGUS	SMM	MM
Serum M-protein	<3.0 g/dL	≥3.0 g/dL	–
Clonal bone marrow plasma cells	<10%	≥10–60%	≥10%
Presence of myeloma-defining event[a]	No	No	Yes

[a]Myeloma-defining events are CRAB criteria (hypercalcemia [serum calcium >0.25 mmol/L (>1 mg/dL) higher than the upper limit of normal or >2.75 mmol/L (>11 mg/dL)], renal insufficiency [serum creatinine >177 µmol/L (2 mg/dL) or creatinine clearance <40 mL/min], anemia [hemoglobin value of >2 g/dL below the lower normal limit, or a hemoglobin value <10 g/dL], bone lesions [one or more osteolytic lesions revealed by skeletal radiography, CT, or PET-CT], or the presence of bone marrow plasma cells >60%, involved/uninvolved free-light chain ratio of ≥100, or >1 focal lesion on MRI [3]

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Table 25.2 Types of monoclonal gammopathy of undetermined significance (MGUS)

Type of MGUS	Definition
Non-IgM MGUS	M-protein <3.0 g/dL Clonal bone marrow plasma cells <10% No myeloma-defining event
IgM MGUS	IgM M-protein <3.0 g/dL Bone marrow lymphoplasmacytic cells <10% No myeloma or Waldenström's macroglobulinemia-defining event
Light chain MGUS	Abnormal free light chain ratio Elevated level of involved light chains No immunoglobulin heavy chain on serum electrophoresis or immunofixation Clonal bone marrow plasma cells <10% No myeloma-defining event No amyloidosis Urine M-protein <500 mg/24 h

MGUS. In the case of progression, non-IgM MGUS in the majority of cases transforms to smoldering myeloma and multiple myeloma [10].

IgM MGUS is less common than non-IgM MGUS and is defined as monoclonal IgM protein <3.0 g/L, <10% bone marrow lymphoplasmacytic cells, and absence of end-organ damage (anemia, hyperviscosity, lymphadenopathy, hepatosplenomegaly, constitutional symptoms) attributed to a lymphoproliferative disorder. Progression from IgM MGUS commonly means transition to smoldering Waldenström's macroglobulinemia or Waldenström's macroglobulinemia but also, although more rare, to IgM multiple myeloma, chronic lymphocytic leukemia or other lymphomas [11]. The *MYD88* mutation is present in the vast majority of Waldenström's macroglobulinemia patients and is found in ~50% of individuals with IgM MGUS [12–14]. IgM multiple myeloma on the other hand is rare, only 1% of all multiple myeloma cases, and is not associated with *MYD88* mutation [12, 15].

Light-chain MGUS (LC-MGUS) was described in 2010 by Dispezieri et al. and represent approximately 20% of all MGUS cases. LC-MGUS is defined as abnormal FLC ratio (<0.26 or >1.65) in combination with elevated concentration of the involved κ or λ light chains [16]. In addition, the definition includes no immunoglobulin heavy chain on serum electrophoresis or immunofixation, <10% clonal bone marrow plasma cells, and no sign of end-organ damage [16, 17]. LC-MGUS can progress to idiopathic Bence Jones proteinuria and light-chain multiple myeloma. Furthermore, LC-MGUS is associated with amyloidosis and renal impairment [16]. Due to the possibility that polyclonal light chains may be elevated in renal disease, Dispenzieri et al. discussed whether using the renal FLC reference range would be more optimal compared to the standard reference for the LC-MGUS definition. According to their estimation, this would affect the number of κ and λ LC-MGUS cases but would not have a major effect on the overall prevalence [16]. This issue was further highlighted by Hutchison et al. who suggested adjusted FLC ratio reference ranges for certain clinical settings [18]. Moreover, polyclonal increase in free light chains can be used as a biomarker for B-cell activation in a broader clinical context. Polyclonal free light chains have been associated with disease activity in autoimmune disease, risk of non-Hodgkin lymphoma, renal impairment, and overall survival in the general population [18]. Further studies are needed to fully elucidate the optimal LC-MGUS definition, polyclonal free light chain increase, and implications for progression to malignant disease.

Epidemiology

Recently, in a large population-based study of the US population, the overall prevalence of MGUS was 2.4% in individuals over the age of 50 years [5]. The study was based on the NHANES and NHANES III studies (2000–2004) which included screening of 12,482 individuals representative for the US population [5]. The highest prevalence of MGUS was found in African-American blacks (3.7%), followed by whites (2.3%), and the lowest prevalence was observed in Mexican-Americans (1.8%) [5]. IgA MGUS was more common among Mexican-Americans and there was a lower rate of IgM MGUS in blacks and Mexican-Americans compared to whites. African-American and African blacks were also more likely to have high-risk features including a higher median level of M-protein and had a higher risk of progression [5, 19]. The overall prevalence of MGUS was lower in the NHANES studies compared to the previous estimates from the Olmsted county studies where the reported prevalence of MGUS was 3.2–3.4% in individuals over 50 years [10, 16]. The difference in reported prevalence may be caused by differences in the patient population, i.e., screened population in the NHANES studies versus referral-based Mayo Clinic cohort [5, 10, 16].

IgM MGUS is less common than non-IgM MGUS but there is limited information on the overall prevalence of IgM MGUS. LC-MGUS comprises 20% of all MGUS cases and was found in 0.7–0.8% of the population over 50 years in studies from the Olmsted County and Germany [16, 20].

The prevalence of MGUS increases with increasing age across all studies. Reported prevalence of MGUS ranged from 1.2 to 2.8% in 50–59-year-olds to 4.6–8.7% in individuals 80 years or older [5, 16, 20]. Moreover, MGUS is consistently more common in men compared to women across all age groups [5].

Etiology

The etiology of both MGUS and multiple myeloma remains largely unknown. The prevalence has been associated with various host factors, e.g., gender, age, and ethnicity, and

there was also a trend towards a higher prevalence of MGUS patients with elevated BMI [5, 19]. First-degree relatives of individuals with MGUS have an increased risk of developing MGUS and LC-MGUS [21–23]. Through genome-wide association studies, a number of single-nucleotide variants associated with an increased risk of multiple myeloma have been identified indicating a genetic susceptibility [24].

A higher risk of developing MGUS has also been reported in individuals exposed to pesticides and herbicides including Agent Orange [6, 25]. Constant immune stimulation may be a trigger of MGUS and an elevated prevalence of MGUS has been observed in patients with a prior autoimmune disorder [26].

Risk Assessment and Progression

Non-IgM MGUS typically progresses to IgG or IgA smoldering myeloma and later to multiple myeloma requiring therapy. The annual risk of progression from MGUS to multiple myeloma is 0.5–1% per year [7, 9]. Patterns of progression can vary from a steady increase in M-protein while others can have a stable M-protein level for many years and then suddenly increase [27]. On average, 75–90% of MGUS patients remain in the precursor stage and never develop malignant disease. However, the risk of progression does not decrease even after 25–35 years and hence lifelong follow-up of MGUS patients is necessary [9, 28]. Patients with smoldering myeloma have a 10% risk of progression per year during the first 5 years after diagnosis and the risk thereafter decreases to around 3% the next 5 years and then 1% per year for the 10 following years [29].

The risk assessment scores in MGUS rely largely on biomarkers. The most updated scoring system was published in 2014 by investigators from the National Cancer Institute and Nordic Myeloma Study Group (NCI/NMSG) [7]. In their population-based cohort followed up to 30 years, the overall risk of progression to multiple myeloma or other lymphoproliferative disorders was 0.5% per year [7]. The rate of progression to malignant disease was higher during the first 10 years after diagnosis, almost 1%/year, and those who remained in the precursor stage after more than 10 years had a lower progression rate. Additionally, by performing new assays on stored samples, they evaluated several biomarkers and the impact on disease progression. In the NCI/NMSG score, a higher risk of transformation to multiple myeloma and lymphoproliferative diseases was observed in patients who had an abnormal FLC ratio (<0.26 or >1.65), M-protein level >1.5 g/dL, and reduction of 1 or 2 noninvolved immunoglobulin isotype levels (immunoparesis) (Table 25.3). In MGUS patients who had all three risk factors, the 10-year risk of disease progression was 40% implying that this group of patients should be carefully monitored for disease progression [7].

Table 25.3 Comparison of the three scoring systems for risk of disease progression in MGUS from the Mayo Clinic, PETHEMA, and NCI/NMSG [7–9]

	NCI/NMSG[a]	Mayo Clinic[b]	PETHEMA[c]
Sample size (*n* MGUS patients)	728	1148	407
Risk factors	Non-IgG MGUS Abnormal FLC ratio M-protein >1.5 g/dL Immunoparesis	Non-IgG MGUS Abnormal FLC ratio M-protein >1.5 g/dL	≥95% aberrant plasma cells DNA aneuploidy
Overall risk of progression	0.5%	1%	~0.6%

FLC free light chain

[a]Population-based cohort with patients included 1964–2000. Score based on unanimously analyzed stored samples

[b]Referral center cohort with patients included between 1960 and 1994. Score based on retrospective laboratory results performed at the time of diagnosis

[c]Referral center cohort with patients included 1996–2003. Score based on flow cytometry performed at the time of diagnosis

Two additional risk scores were published in 2005 and 2007 by the Mayo Clinic and PETHEMA groups, respectively [8, 9]. The Mayo Clinic risk score is based on the type and size of M-protein as well as serum FLC ratio. High-risk criteria are defined as non-IgG M-protein, M-protein >1.5 g/dL, and an abnormal FLC ratio [9]. In this system, high-risk MGUS patients who have all the three risk factors had a cumulative risk of developing multiple myeloma of 58% during the first 20 years after the MGUS diagnosis. Patients with two, one, and no risk factors have a risk of progression of 37%, 21%, and 5%, respectively [10]. The PETHEMA group bases their prognostic system on aberrant plasma cells in the bone marrow and DNA aneuploidy both measured by flow cytometry. MGUS patients who had ≥95% aberrant plasma cells and DNA aneuploidy had a 5-year risk of progression of 46%, while individuals with one of the two risk factors had a 10% risk of progression and patients with no risk factor a 2% risk of progression at 5 years, respectively [8].

Additional markers associated with a higher risk of progression from MGUS to multiple myeloma are suppression of nonclonal bone marrow plasma cells, similar to the above-mentioned immunoparesis [7, 8, 30, 31]. A progressive increase in the M-protein level is also a prognostic marker of progression [32]. Different rates of progression were reported for IgG MGUS vs. non-IgG MGUS in the Mayo Clinic model while no such difference was seen in the NCI/NMSG and PETHEMA models. The underlying reason for this difference is not fully understood but may be related to the higher catabolic rate of IgA and IgM compared to IgG implying that at a given M-protein in serum, there may be more clonal plasma cells in the bone marrow in individuals with IgA and IgM isotype MGUS compared to those with IgG MGUS [7,

8]. Furthermore, gene expression profiling can additionally contribute to the risk assessment in MGUS and smoldering myeloma [33]. Presence of circulating plasma cells, abnormal metaphase cytogenetics, and cytoplasmic immunoglobulin measured by flow cytometry can also be valuable in predicting disease progression [34, 35]. It is on the other hand not clear if cytogenetic markers of poor risk in multiple myeloma, t(4;14) or del(17p), are associated with an increased risk of malignant transformation in MGUS patients [36].

The risk of progression from IgM MGUS to Waldenström's macroglobulinemia or other lymphoproliferative diseases is approximately 1.5% per year [37]. Risk factors for progression in IgM MGUS include M-protein size >1.5 g/dL and presence of *MYD88* mutation. Abnormal FLC ratio is not an established risk factor for progression and is not included in the recommended follow-up of patients with IgM MGUS [12, 14].

There is so far limited information on the risk of progression in LC-MGUS. Based on the two published studies so far, the risk of progression appeared to be lower than 1% per year but more information is needed in order to give an accurate estimate [16, 20]. In addition to light-chain multiple myeloma, patients with LC-MGUS are at an elevated risk of developing amyloidosis and renal disease. The latter was observed in 23% of patients and the majority of these had λ-restricted LC-MGUS [16].

The prognostic factors and existing scoring systems are valuable for risk stratification but there is a need to identify molecular markers to better predict the risks in the individual patient.

Genetic Background

Patients with multiple myeloma can be classified into two major cytogenetic groups: chromosome 14 translocations (*IGH* locus) and hyperdiploidy. In studies using fluorescent in situ hybridization (FISH), these cytogenetic aberrations were present already in MGUS and are thus considered early hits in myelomagenesis [38].

During recent years, knowledge of the genetic landscape in multiple myeloma has increased greatly through studies using modern sequencing techniques, i.e., whole-genome sequencing, whole-exome sequencing, and targeted sequencing. So far, no single disease-specific gene has been identified; the studies on the contrary revealed a complex genomic landscape including frequent somatic mutations in *KRAS*, *NRAS*, *FAM46C*, *BRAF*, *TP53*, *TRAF3*, *DIS3*, *CYLD*, and more [39–42]. There is so far limited information on driver mutations and changes in the genomic landscape during transition from precursor to malignant disease. Progression from MGUS to multiple myeloma may be caused by acquisition of additional genetic events or the expansion of preexisting clones already present at the MGUS stage. In both scenarios, interactions between the plasma cells and the bone marrow microenvironment as well as the immune system are likely to influence disease evolution [43].

Comorbidities

Through assessment of large clinical cohorts, MGUS has been associated with a number of comorbidities and mortality not only associated with the development of multiple myeloma. These include increased risk of infections, venous and arterial thrombosis, malignancies, and an inferior survival compared to the general population [21, 44, 45]. Furthermore, patients with MGUS have an increased bone turnover and an elevated risk of fractures [46, 47]. In screened cohorts, the rate of these complications tended to be lower indicating a possible role of other underlying comorbidities which may have led to the original clinical workup and diagnosis of MGUS [48, 49].

There is an increased risk of renal complications in patients with MGUS, especially LC-MGUS, and the term monoclonal gammopathy of renal significance has therefore been coined [50]. M-protein-related renal diseases include monoclonal immunoglobulin deposition disease, light-chain proximal tubulopathy, proliferative glomerulonephritis with monoclonal immunoglobulin deposits, and C3 glomerulopathy with monoclonal gammopathy. These renal diseases are characterized by deposition of monoclonal deposits in the kidney diagnosed on kidney biopsy [36, 50]. In addition, the M-protein can cause various systemic manifestations including neuropathy and skin disorders [36, 43].

Clinical Recommendations

The recommended workup for MGUS is aimed at detecting end-organ damage indicative of multiple myeloma, lymphoproliferative disorders, or amyloidosis. Workup recommended by the International Myeloma Working Group includes complete blood counts, electrolytes and renal function test, serum and urine electrophoresis, as well as serum FLC assay (Table 25.4) [3, 51]. Bone marrow biopsy and bone imaging are recommended to confirm the MGUS diagnosis and to rule out CRAB criteria of other findings of smoldering or multiple myeloma. In patients with low-risk MGUS, bone marrow biopsy and imaging can be deferred if there are no signs of high-risk MGUS, or end-organ damage indicating multiple myeloma, lymphoma, or amyloidosis [51, 52]. If amyloidosis is suspected, biopsy of the bone marrow or abdominal fat with Congo red staining should be performed [36].

Patients diagnosed with MGUS should be monitored every 4–6 months during the first year after initial diagnosis. Thereafter, evaluation every 6–12 months is recommended depending on the rate of M-protein increase. In patients with stable low-risk MGUS, evaluation every 24 months is sufficient while closer monitoring is recommended in high-risk MGUS patients [36, 52]. Patients with LC-MGUS should be evaluated regularly for development of kidney disease and amyloidosis with NT-pro-BNP and urine albumin.

Table 25.4 Clinical evaluation for newly diagnosed patients with monoclonal gammopathy of undetermined significance (MGUS)

• Complete blood count
• Chemistry including serum electrolytes, creatinine, beta-2 microglobulin, calcium, albumin, lactate dehydrogenase (LDH)
• Serum protein studies including total protein and serum electrophoresis
• Serum immunofixation
• Serum free light chain measurements
• 24-h urine collection for electrophoresis and immunofixation
• Bone marrow aspirate and biopsy for plasma cell infiltration, flow cytometry, and fluorescence in situ hybridization[a]
• Skeletal survey, low-dose CT, or PET-CT[a]

[a]These assessments can be excluded in patients who are considered low-risk MGUS (IgG MGUS, M-protein <1.5 g/dL, and normal free light chain ratio)

Recently, two independent studies have reported a better overall survival in multiple myeloma patients where the MGUS was previously known compared to multiple myeloma patients without prior knowledge of MGUS [53, 54]. This is likely an effect of earlier diagnosis and treatment and raises the question whether screening for MGUS would be beneficial. There are ongoing studies for earlier treatment of patients with smoldering myeloma, especially those with high risk, but so far treatment for patients with MGUS is not recommended.

Conclusion

MGUS is a premalignant disorder that in the majority of cases is asymptomatic and will not progress to malignant disease. The risk of progression to malignant disease persists throughout life and patients should therefore be monitored continuously. Thorough workup is needed to exclude more advanced disease stages and associated disorders. There are valuable risk-scoring systems for predicting the risk of transformation to multiple myeloma and there are ongoing research efforts to identify molecular markers that can better predict individual risk of progression.

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26 Smoldering Multiple Myeloma

María-Victoria Mateos and Jesús F. San-Miguel

Introduction

Smoldering multiple myeloma (SMM) is an asymptomatic plasma cell disorder defined in 1980 by Kyle and Greipp on the basis of a series of six patients who met the criteria for multiple myeloma (MM) but whose disease did not have an aggressive course [1].

At the end of 2014, the International Myeloma Working Group (IMWG) updated the definition and SMM was defined as a plasma cell disorder characterized by the presence of ≥3 g/dL serum M-protein and/or 10–60% bone marrow plasma cells (BMPCs), but with no evidence of myeloma-related symptomatology (hypercalcemia, renal insufficiency, anemia, or bone lesions (CRAB)) or any other myeloma-defining event (MDE) [2]. According to these recent updated criteria, the definition of SMM excludes asymptomatic patients with BMPCs of 60% or more, serum free light chain (FLC) levels of ≥100, and those with two or more focal lesions in the skeleton as revealed by magnetic resonance imaging (MRI).

Kristinsson et al., based on the Swedish Myeloma Registry, have recently reported that 14% of patients diagnosed with myeloma had SMM and, accordingly, the age-standardized incidence of SMM would be 0.44 cases per 100,000 people [3].

Differential Diagnosis with Other Entities

SMM must be distinguished from other plasma cell disorders, such as monoclonal gammopathy of undetermined significance (MGUS) and symptomatic MM (Table 26.1). The MGUS entity is characterized by a level of serum M-protein of <3 g/dL plus <10% plasma cell infiltration in the bone marrow, with no CRAB and no MDE. Symptomatic MM must always have CRAB symptomatology or MDE, in conjunction with ≥10% clonal BMPC infiltration or biopsy-proven bony or extramedullary plasmacytoma [2].

End-organ damage often needs to be correctly evaluated to distinguish myeloma-related symptomatology from some signs or symptoms that could otherwise be attributed to comorbidities or concomitant diseases [4].

Due to the updated IMWG criteria for the diagnosis of MM, there are some specific assessments to which physicians have to pay attention in order to make a correct diagnosis of SMM [2].

1. For evaluation of bone disease, the IMWG recommends to perform in all patients with suspected SMM one of the following procedures: skeletal survey, ^{18}F-fluorodeoxyglucose

Table 26.1 Differential diagnosis of MGUS, SMM, and symptomatic MM

Feature	MGUS	SMM	MM
Serum-M protein	<3 g/dL and	≥3 g/dL and/or	
Clonal BMPC infiltration	<10%	10–60%	≥10% or biopsy-proven plasmacytoma
Symptomatology	Absence of CRAB[a]	Absence of MDE[b] or amyloidosis	Presence of MDE[b]

[a]CRAB includes (1) hypercalcemia: serum calcium >0.25 mmol/L (>1 mg/dL) higher than the upper limit of normal or >2.75 mmol/L (>11 mg/dL); (2) renal insufficiency: serum creatinine >177 μmol/L (2 mg/dL) or creatinine clearance <40 mL/min; (3) anemia: hemoglobin value of >2 g/dL below the lower normal limit, or a hemoglobin value <10 g/dL; (4) bone lesions: one or more osteolytic lesions revealed by skeletal radiography, CT, or PET-CT

[b]*MDE* Myeloma-defining events include CRAB symptoms (above) or any one or more of the following biomarkers of malignancy: clonal bone marrow plasma cell percentage ≥60%; involved/uninvolved serum free light chain ratio ≥100; >1 focal lesions revealed by MRI studies

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(FDG) positron emission tomography (PET)/computed tomography (CT), or low-dose whole-body CT, with the exact modality determined by availability and resources. The aim is to exclude the presence of osteolytic bone lesions, currently defined by the presence of at least one lesion (≥5 mm) revealed by X-ray, CT, or PET-CT. In addition, whole-body MRI of the spine and pelvis is a mandatory component of the initial workup. It provides detailed information about not only bone marrow involvement but also the presence of focal lesions that predict more rapid progression to symptomatic myeloma. Hillengass et al. reported in 2010 that the presence of more than one focal lesion in whole-body MRI was associated with a significantly shorter median time to progression (TTP) to active disease (13 months), as compared to patients without focal lesions [5]. Kastritis and colleagues reported similar results after the analysis of a subgroup of patients who underwent spinal MRI and were followed up for a minimum of 2.5 years. The median TTP to symptomatic disease was 14 months when more than one focal lesion was present [6]. Therefore, if more than one focal lesion in MRI is present in SMM patients, this entity should no longer be considered as SMM but as MM, according to the current IMWG criteria. It is important to emphasize that they should be unequivocal focal lesions of >5 mm.

2. With respect to bone marrow infiltration, the Mayo Clinic group evaluated BMPC infiltration in a cohort of 651 patients and found that 21 (3.2%) had an extreme infiltration (≥60%) [7]. This group of patients had a median TTP to active disease of 7.7 months, with a 95% risk of progression at 2 years. This finding was subsequently validated in a study of 96 patients with SMM, in whom a median TTP of 15 months was reported for the group of patients with this extreme infiltration [8]. In a third study, 6 of 121 patients (5%) with SMM were found to have ≥60% BMPC, and all progressed to MM within 2 years [9]. Therefore, if ≥60% of clonal plasma cell infiltration is present either in bone marrow aspirate or biopsy, the diagnosis of SMM should be replaced by MM. Additional assessments, for example, by flow cytometry or by identifying cytogenetic abnormalities in SMM patients, are not mandatory but can help to estimate the risk of progression to active disease.
3. With respect to the serum free light chain (FLC) assay, Larsen et al. studied 586 patients with SMM to determine whether there was a threshold FLC ratio that predicted 85% of progression risk at 2 years. They found a serum-involved/uninvolved FLC ratio of at least 100 in 15% of patients and their risk of progression to symptomatic disease was 72% [10]. Similar results were obtained in a study by Kastritis and colleagues from the Greek Myeloma Group. In their study of 96 SMM patients, 7% had an involved/uninvolved FLC ratio of ≥100 and almost all progressed within 18 months [8]. In a third study, the risk of progression within 2 years was 64% [9]. Therefore, physicians must perform the sFLC assay at the moment SMM is first suspected and, if the involved/uninvolved ratio is ≥100, a diagnosis of active MM instead of SMM should be established.

Table 26.2 Workup for newly diagnosed SMM patients

• Medical history and physical examination
• Hemogram
• Biochemical studies, including creatinine and calcium levels; beta-2 microglobulin, LDH, and albumin
• Protein studies
– Total serum protein and serum electrophoresis (serum M-protein)
– 24-h urine sample protein electrophoresis (urine M-protein)
– Serum and urine immunofixation
• Serum free light chain measurement (sFLC ratio)
• Bone marrow aspirate ± biopsy: infiltration by clonal plasma cells, flow cytometry, and fluorescence in situ hybridization analysis
• Skeletal survey, CT, or PET-CT
• MRI of thoracic and lumbar spine and pelvis; ideally, whole-body MRI

FLC free light chain, *CT* computed tomography, *PET-CT* ^{18}F-fluorodeoxyglucose (FDG) positron emission tomography (PET)/CT, *MRI* magnetic resonance imaging

If, after considering the specific assessments mentioned above (Table 26.2), a diagnosis of SMM is finally made, the serum and urine M-component, hemoglobin, calcium, and creatinine levels should be reevaluated 2–3 months later in order to confirm the stability of these parameters. The frequency of the subsequent follow-up exams should be adapted on the basis of risk factors for progression to symptomatic MM (see below).

How to Evaluate the Risk of Progression to MM?

The annual risk of progression from SMM to symptomatic MM is 10% per year for the first 5 years, 5% per year during the following 5 years, and only 1% per year after 10 years [11]. Though most patients diagnosed with SMM will progress to symptomatic MM and will need to start treatment, SMM is not a uniform disorder.

Several groups have reported possible predictors of progression to symptomatic MM, and this information could be useful for physicians and can help to explain to patients their risk of progression to active MM (Table 26.3).

– Size of serum M-protein and the extent of marrow involvement

Table 26.3 Smoldering MM: markers predicting progression to symptomatic MM

Features for identifying high-risk SMM patients: 50% at 2 years
• Tumor burden:
– ≥10% clonal plasma cell bone marrow infiltration plus
– ≥3 g/dL of serum M-protein and
– Serum free light chain ratio between 0.125 and 8
– Bence Jones proteinuria positive from 24-h urine sample
– Peripheral blood circulating plasma cells >5 × 10^6/L
– Peripheral blood circulating plasma cells ≥150 by flow cytometry
• Immunophenotyping characterization and immunoparesis:
– ≥95% of aberrant plasma cells by flow within the plasma cell bone marrow compartment plus
– Immunoparesis (>25% decrease in one or both uninvolved immunoglobulins relative to the lowest normal value)
• Cytogenetic abnormalities:
– Presence of t(4;14)
– Presence of del (17p)
– Gain of 1q21
– Hyperdiploidy
– Gene expression profiling risk score >−0.26
• Pattern of serum M-component or hemoglobin evolution:
– Evolving type: If M-protein ≥3 g/dL, increase of at least 10% within the first 6 months. If M-protein <3 g/dL, annual increase of M-protein for 3 years
– Increase in the M-protein to ≥3 g/dL over the 3 months since the previous determination
– Decrease of hemoglobin in ≥0.50 g/dL within 12 months of diagnosis
• Imaging assessments:
– MRI: Radiological progressive disease (MRI-PD) was defined as newly detected focal lesions (FLs) or increase in diameter of existing FL and a novel or progressive diffuse infiltration
– Positive PET/CT with no underlying osteolytic lesion

MRI magnetic resonance imaging, *PET-CT* ^{18}F-fluorodeoxyglucose (FDG) positron emission tomography (PET)/CT

– Mayo Clinic group [11] proposed three SMM subgroups according to BMPC infiltration and the size of the serum M-protein. Group 1 was characterized by ≥3 g/dL of M-protein and ≥10% of BMPCs, with a median TTP to symptomatic MM of 2 years. Group 2 featured ≤3 g/dL of M-protein and ≥10% BMPCs with a median TTP of 8 years. Group 3 had ≥3 g/dL of M-protein but <10% BMPC infiltration, resulting in a median TTP of 19 years.

– Serum free light chain ratio

– The Mayo Clinic group also evaluated the previously described patient population to identify the risk of progression to symptomatic myeloma on the basis of a free light chain (FLC) assay. A kappa/lambda FLC ratio between 0.125 and 8 was found to be associated with an increased risk of progression to symptomatic MM. This parameter was added to their previous score, which considered the size of serum M-protein and BMPC infiltration, to refine the Mayo risk stratification model. This yielded three groups, with a median TTP of 1.9 years for the high-risk group, whose members exhibited all three defined risk factors [12].

– The Danish Myeloma group did not found in the analysis of their registry any significant threshold for the serum free light chain ratio; therefore they do not support the recent IMWG proposal that identifies patients with a FLC ratio above 100 as having ultrahigh risk of transformation to MM [13].

– Immunophenotyping and immunoparesis

– Multiparameter flow cytometry (MFC) to identify the immunophenotypic profile of plasma cells in SMM has been evaluated by the Spanish Myeloma group. We reported that the presence of an aberrant BMPC phenotype in the vast majority of PC (≥95% phenotypically abnormal plasma cells from total PC), determined by MFC (defined as the overexpression of CD56 and CD19, CD45 negative, and/or decreased reactivity for CD38), was the most important predictor of early progression from SMM to active MM [14]. The presence of immunoparesis (i.e., a decrease in one or two of the uninvolved immunoglobulins to 25% below the lowest normal value) also emerged as a significant independent prognostic characteristic. Based on these two parameters, the Spanish group proposed a scoring system that stratified SMM patients into three categories with a median TTP of 23 months when the two risk factors were present, compared with 73 months when only one was present, and not reached when neither was present [15].

– The Danish Myeloma group has recently reported that both an M-protein ≥3 g/dL and immunoparesis significantly influenced TTP (HR 2.7 95% CI(1.5;4.7) $p = 0.001$ and HR 3.3 95%CI(1.4;7.8) $p = 0.002$, respectively) to myeloma [13].

– Peripheral blood circulating plasma cells

– The Mayo Clinic group has also evaluated the role of peripheral blood circulating PCs in 171 SMM patients, and in those (15%) who had high levels of circulating PCs (>5 × 10^6/L and/or >5% PCs per 100 cytoplasmic immunoglobulin (Ig)-positive mononuclear cells), the progression risk at 2 years was significantly higher than for patients with low levels of circulating PC (71% vs. 24%; $p = 0.001$) [16]. This group has recently improved the identification of peripheral blood circulating plasma cells in SMM using flow cytometry in 100 patients. The median TTP of patients with 150 or more circulating PCs was 9 months compared to not reached for patients with less than 150 circulating PCs ($P < 0.001$). In the

future, this may allow reclassification of such patients as having MM requiring therapy prior to them enduring end-organ damage [17].

- Pattern of serum M-component evolution
- The pattern of evolution of the monoclonal component during the course of the disease enabled to identify two types of SMM: evolving and nonevolving. Based on the analysis of 207 SMM patients, the evolving type was defined by the following criteria: (1) if the concentration of M-protein was ≥3 g/dL at baseline, the evolving type featured an increase in M-protein of at least 10% within the first 6 months following diagnosis; (2) if the concentration of M-protein was <3 g/dL at baseline, the evolving type featured a progressive increase in M-protein in each consecutive annual measurement over a 3-year period [18]. The evolving pattern was recognized in 25% of patients, and was associated with a probability of progression of 45% at 2 years, with a median TTP to active MM of 3 years, compared with 19 years for those with the nonevolving type [19]. The Mayo Clinic group has recently validated the evolving change in the monoclonal protein (eMP) in a series of 191 patients with SMM, resulting in an odds ratio of 7.26 (2.89–18.26, $p < 0.001$); moreover, they found the BMPC infiltration >20% and the evolving change in hemoglobin (eHb), defined as ≥0.5 g/dL decrease within 12 months of diagnosis, as independent prognostic markers predicting progression and a new risk model comprising these variables was constructed, with median TTP of 12.3, 4.2, 2.8, and 1.0 years in patients with none, 1, 2, and 3 risk factors, respectively ($p < 0.001$). The 2-year risk of progression was 82.8% in patients with both eMP and eHb, and increased to 90.9% in those with all three risk factors. This new risk model would identify patients with SMM candidates to be considered in the future as MM [20]. The SWOG group also found that patients with an increase in the M-component ≥3 g/dL over the 3 months since their previous determination had an associated risk of progression of approximately 50% at 2 years [21].
- Bence Jones proteinuria
- One hundred and forty-seven SMM patients were examined for the presence of Bence Jones proteinuria at diagnosis, and its effect on progression to symptomatic disease was assessed. The study showed that in SMM patients in which the M-protein was defined by a complete immunoglobulin, but who were also positive for Bence Jones proteinuria, regardless of the amount, the risk of progression to active disease was significantly higher than in Bence Jones proteinuria-negative patients (22 vs. 83 months; $p < 0.001$). In addition, when Bence Jones proteinuria in the 24-h urine sample exceeded 500 mg, the risk was even higher, with a median TTP of 7 months indicating that this parameter would be a new biomarker to identify SMM patients that could be considered as MM [22].
- Novel imaging assessments
- The novel imaging assessments have contributed to the updated criteria for the definition of MM and SMM, as has been previously mentioned. However, the new imaging assessments can also help to predict progression risk in SMM. The first studies with spinal MRI were done in SMM patients and the presence of a focal pattern was associated with a shorter TTP as compared to that of a diffuse or variegated pattern (median 6 vs. 16 vs. 22 months). Hillengass et al. have recently evaluated the role of MRI during the follow-up of patients with SMM. Radiological progressive disease (MRI-PD), which they defined as the detection of new focal lesions or the increase in diameter of existing focal lesions, and a novel or progressive diffuse infiltration, was identified as a feature for classifying SMM patients at high risk of progression to symptomatic disease [23]. The role of PET/CT has also been evaluated in SMM. The Italian group has recently reported that approximately 10% of SMM patients from a series of 73 patients had a positive result with PET/CT with no underlying osteolytic lesion, and this predicted for high risk of progression to symptomatic disease (48% at 2 years compared with 32% for PET/CT-negative patients; $p = 0.007$) [24]. The Mayo Clinic group also identified a subgroup within a series of 132 SMM patients who showed a positive result with PET/CT in which the rate of progression to MM within 2 years was 56%, as compared to 28% among PET/CT-negative patients ($p = 0.001$). The rate of progression was even higher among patients on whom PET/CT was performed within 3 months of their diagnosis of SMM (74% vs. 27% in PET/CT-negative patients) [25].
- Cytogenetic abnormalities
- The Mayo Clinic group analyzed the cytogenetic abnormalities in a series of 351 SMM patients and identified a high-risk subgroup of patients with t(4;14) and/or del(17p) with a significantly shorter median TTP (24 months) as compared to the intermediate-, standard-, and low-risk patient subgroups [26]. The high risk of progression of SMM to MM with t(4;14) may be related to the fact that this abnormality is associated with markedly high FLC ratios. However, the mechanism by which a high FLC ratio is associated with a higher risk of progression is not clear and is only partly related to renal failure from cast nephropathy. Neben et al. have identified t(4;14), gain of 1q21, or hyperdiploidy as being independent prognostic factors for a shorter TTP. The median TTP for patients with del(17p) was 2.7 years (vs. 4.9 years for those without the translocation; $p = 0.019$), 2.9 years for patients

with t(4;14) (vs. 5.2 years for those without the translocation; $p = 0.021$), and 3.7 years for patients with gain of 1q21 (vs. 5.3 years for those without the gain; $p = 0.013$). In addition, hyperdiploidy was associated with a significantly shorter median TTP of 3.9 years (compared with 5.7 years for non-hyperdiploid patients; $p = 0.036$) [27].

Finally, the South West Oncology Group (SWOG) evaluated the Gene Expression Profiling 40 (GEP40) model in a group of 105 SMM patients. A gene signature derived from four genes, at an optimal binary cut point of 9.28, identified 14 patients (13%) with a 2-year progression risk of 85.7%. Conversely, a low four-gene score (< 9.28) combined with baseline monoclonal protein <3 g/dL and albumin ≥3.5 g/dL identified 61 patients with low-risk SMM with a 5.0% risk of progression at 2 years [28]. Landgren et al. have recently performed whole exome sequencing and RNA sequencing in 12 patients with high-risk SMM and 39 patients with newly diagnosed symptomatic MM. Despite having only a few high-risk SMM patients in this study, none of them had any mutations in the recurrently mutated genes found in the symptomatic MM group, indicating that the molecular profile of SMM could be different. Therefore, these findings might have some implications for risk assessment and initiation of therapy [29].

In summary, the diagnosis of SMM is associated with a variable risk of progression to active disease, and the presence of the aforementioned prognostic factors can discriminate subgroups of patients based on their degree of risk (Table 26.3).

Stratification and Management of SMM Patients

The first step in clinical practice is to identify the risk of progression to active disease for each newly diagnosed SMM patient. The key question is which risk model is better for evaluating the risk of progression to symptomatic disease for each individual SMM patient. Both the Mayo Clinic and Spanish models have been validated in a prospective trial. However, new risk models are emerging that incorporate novel clinical and biological features [9, 11, 13, 15, 18, 21, 27, 30, 31] (Table 26.4). The components of these models are not identical, and each patient's risk should probably be defined on the basis of all the available data rather than through the use of a restricted model (Table 26.3). These models identified their risk factors as independent variables in multivariate analysis. Some of the features evaluated in each risk model can overlap, but not all of them have to be present in a SMM patient to be defined as a high-risk SMM patient.

Table 26.4 Risk models for the stratification of SMM

Risk model	Risk of progression to MM	
Mayo Clinic		*Median TTP*
– ≥10% clonal PCBM infiltration – ≥3 g/dL of serum M-protein – Serum FLC ratio between <0.125 and >8	1 risk factor 2 risk factors 3 risk factors	10 years 5 years 1.9 years
Spanish myeloma		*Median TTP*
– ≥95% of aberrant PCs by MFC – Immunoparesis	No risk factor 1 risk factor 2 risk factors	NR 6 years 1.9 years
Heidelberg		*3-year TTP*
– Tumor mass using the Mayo model – t(4;14), del17p, or +1q	T-mass low + CA low risk T-mass low + CA high risk T-mass high + CA low risk T-mass high + CA high risk	15% 42% 64% 55%
SWOG		*2-year TTP*
– Serum M-protein ≥2 g/dL – Involved FLC >25 mg/dL – GEP risk score >–0.26	No risk factor 1 risk factor ≥2 risk factors	30% 29% 71%
Penn		*2-year TTP*
– ≥40% clonal PCBM infiltration – sFLC ratio ≥50 – Albumin ≤3.5 mg/dL	No risk factor 1 risk factor ≥2 risk factors	16% 44% 81%
Japanese		*2-year TTP*
– Beta-2 microglobulin ≥2.5 mg/L – M-protein increment rate >1 mg/dL/day	2 risk factors	67.5%
Czech and Heidelberg		*2-year TTP*
– Immunoparesis – Serum M-protein ≥2.3 g/dL – Involved/uninvolved sFLC >30	No risk factor 1 risk factor 2 risk factors 3 risk factors	5.3% 7.5% 44.8% 81.3%
Barcelona		*2-year TTP*
– Evolving pattern = 2 points – Serum M-protein ≥3 g/dL = 1 point – Immunoparesis = 1 point	0 points 1 point 2 points 3 points	2.4% 31% 52% 80%
Mayo Clinic evolving model		
– eMP – eHB – ≥20% plasma cells	0 points 1 point 2 points 3 points	12.3 years 4.2 years 2.8 years 1 year
Danish		*3-year TTP*
– Serum M-protein ≥3 g/dL – Immunoparesis	No risk factor 1 risk factor 2 risk factors	5% 21% 50%

SMM patients should be classified as follows:

1. Patients at low risk of progression who are characterized by the absence of the aforementioned high-risk factors (using the validated Mayo or the Spanish risk models), with a probability of progression at 5 years of only 8%. These patients behave similarly to MGUS-like patients and should be followed annually.
2. The second group includes patients at intermediate risk of progression and they only display some of the aforementioned high-risk factors. These are probably the true SMM patients. They have a risk of progression at 5 years of 42%, and they must be followed up every 6 months (except during the first year that should be followed every 3–4 months in order to exclude an SMM evolving form).
3. The third group includes high-risk patients classified on the basis of one of the risk models mentioned before. Half of them will progress during the 2 years following diagnosis. These group of patients need a close follow-up every 2–3 months. As there is not any treatment approved yet for these high-risk SMM patients, the best approach should be to refer them to specialized centers in MM therapy and to include them in clinical trials to better understand their biology and to confirm the survival benefit of early treatment in this cohort [32].

The Spanish myeloma group (GEM/Pethema) conducted a phase 3 randomized trial in 119 SMM patients at high risk of progression to active disease (according to the Mayo and/or Spanish criteria). This trial compared early treatment with lenalidomide plus dexamethasone as induction followed by lenalidomide alone as maintenance versus observation. The primary end point was TTP to symptomatic MM, and after a median follow-up of 40 months, the median TTP was significantly longer in patients in the early treatment group than in the observation arm (not reached vs. 21 months; hazard ratio, HR = 5.59; $p < 0.001$). This trial has been recently updated, and after a median follow-up of 75 months, lenalidomide plus dexamethasone continued to provide a benefit in terms of TTP compared with observation (median TTP not reached (95% CI 47 months–not reached) vs. 23 months (95% CI 16–31 months); hazard ratio (HR) 0.24 (95% CI (0.14–0.41); $p < 0.0001$)). Progression to multiple myeloma occurred in 53 (86%) of 62 patients in the observation group compared with 22 (39%) of 57 patients in the treatment group. At data cutoff, 10 (18%) patients had died in the treatment group and 22 (36%) patients had died in the observation group; median overall survival from the time of study entry had not been reached in either group (HR 0.43 (95% CI 0.21–0.92), $p = 0.024$)) [33]. The safety profile was acceptable and most of the adverse events reported were grade 1 or 2. This study showed for the first time the potential for changing the treatment paradigm for high-risk SMM patients based on the efficacy of early treatment in terms of TTP to active disease and of OS, confirmed after long-term follow-up. Moreover, several trials currently under way are investigating the role, on high-risk SMM patients, of novel agents such as lenalidomide alone, siltuximab (anti-IL6 monoclonal antibody), elotuzumab (anti-SLAMF7 monoclonal antibody), or lenalidomide-dexamethasone plus elotuzumab. Promising efficacy results have been reported for the combination of lenalidomide plus dexamethasone with the novel proteasome inhibitor carfilzomib in a series of 12 high-risk SMM patients. All patients achieved CR and most were in immunophenotypic CR [34]. The next step will be to develop a more intensive therapeutic approach for young high-risk SMM patients, similar to the treatment planned for young symptomatic MM patients, for whom "cure" should be the objective.

Conclusion and Future Directions

The treatment philosophy for MM patients has mainly focused on symptomatic patients. This approach is clearly different from those adopted to treat other malignancies, such as breast, colon, or prostate cancer, for which early intervention is not only appropriate, but also essential for success and cure. This difference in philosophy arose for several reasons: (1) in the past, only a few drugs, most of which were alkylating agents, were available to treat MM; (2) the trials conducted in asymptomatic MM patients failed to produce a significant benefit; and (3) the risk of progression to active disease in SMM patients is relatively low (10% per year).

However, significant advances are being made in the understanding and management of SMM patients. From the biological point of view, different subgroups of SMM patients have been identified, including those patients with >60% PC or FLC ratio >100 or two or more focal lesions, that are now considered as active MM patients in which treatment should be started before myeloma-related symptoms develop. In the near future, new biomarkers will be considered to expand the inclusion of SMM patients at imminent risk of progression to active disease in order to be considered as MM.

Moreover, we will soon have the results from several current trials conducted in high-risk SMM patients, which will enable us to offer early treatment for a selected group of asymptomatic myeloma patients with the confidence that some of them will be "cured." The cure-versus-control debate is particularly pertinent in asymptomatic myeloma patients. Some physicians argue in favor of controlling the disease through continuous oral therapy mainly based on immunomodulatory agents, while others support the intensive therapy approaches, including high-dose therapy and transplant, with the objective of eradicating the disease.

Ongoing biological studies will also help us to better understand the pathogenesis of the disease and to identify the key drivers of the transition from monoclonal gammopathy to smoldering and symptomatic disease. These drivers may represent optimal targets for new therapeutic approaches.

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27 Frail Patients with Newly Diagnosed Multiple Myeloma

Alessandra Larocca and Antonio Palumbo

Introduction

Multiple myeloma (MM) is a neoplastic disease deriving from an abnormal proliferation of monoclonal plasma cells in the bone marrow. MM is characterized by both intrinsic genetic alterations in the clonal plasma cells and microenvironmental changes [1].

This disease accounts for 13% of all hematological cancers, approximately 2% of all cancer deaths, and 20% of deaths caused by hematological malignancies. The annual age-adjusted incidence is 5.6 cases per 100,000 people in Western countries [1]. MM is typical of the elderly, median age at diagnosis is approximately 70 years, 37% of patients at diagnosis are less than 65 years, 27% are aged 66 to 75 years, and 37% of patients are over 75 years.

Of note, life expectancy is increasing worldwide. Indeed, the global population is rapidly aging, and the number of individuals aged ≥65 years is expected to double between 2000 and 2030. As a consequence, also the prevalence of myeloma is likely to increase [2].

The diagnosis of MM requires the presence of at least 10% of monoclonal plasma cells at the bone marrow biopsy. Myeloma is defined asymptomatic (smoldering), when no myeloma-related organ or tissue dysfunction is present, or symptomatic, when the so-called CRAB criteria are present: C: hypercalcemia (>11.5 mg/dL); R: renal failure (serum creatinine >1.73 mmol/L); A: anemia (hemoglobin <10 g/dL or >2 g/dL below the lower limit of normal); and B: bone disease (lytic lesions, severe osteopenia, or pathologic fractures) [3]. The diagnostic criteria have been recently updated, and the presence of more than 60% of monoclonal plasma cells in the bone marrow, or one or more focal lesions detected with magnetic resonance imaging (MRI), or an abnormal ratio of the serum free light chains have been added as a clinically relevant criteria to start treatment [4]. To date, treatment is started in patients with symptomatic disease, whereas only clinical observation is recommended in patients with asymptomatic myeloma. Yet, ongoing trials are evaluating the role of novel agents in delaying the progression from asymptomatic to active disease.

Treatment strategy for MM has long been based on patients' age. Patients aged 65–75 years are generally considered ineligible for autologous stem cell transplantation (ASCT). Biologic age does not always correspond to chronologic age, and this strict range may differ by approximately 5 years [5]. In Europe, current treatment for elderly patients older than 65 years or younger with significant comorbidities and ineligible for ASCT consists of the combinations of melphalan-prednisone plus either the immunomodulatory agent thalidomide (MPT) or the proteasome inhibitor bortezomib (VMP) [6, 7]. Recently the European and American regulatory authorities approved lenalidomide in combination with dexamethasone in the United States and in combination with dexamethasone or with melphalan and prednisone followed by lenalidomide monotherapy maintenance for ASCT-ineligible patients in Europe [8, 9]. In the United States, age is not the primary criterion to establish whether a patient is eligible for ASCT, and patients who are not candidates for ASCT can receive initial therapy with either bortezomib-lenalidomide-dexamethasone (VRD), bortezomib-cyclophosphamide-dexamethasone (VCD), or also Rd [10].

Usually, gentler approaches are used for patients older than 75 years, who may receive reduced-dose treatments based solely on their age [11].

Survival of MM patients varies greatly, from less than 6 months to more than 10 years, depending on both biological features of the disease at diagnosis (albumin,

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beta-2-microglobulin, and cytogenetics) and patient characteristics (age, performance status, comorbidities). In patients younger than 65 years eligible for ASCT, the current expected overall survival (OS) is 5–7 years; in patients ineligible for ASCT undergoing conventional chemotherapy it is 3–4 years [1].

The introduction of novel agents thalidomide, lenalidomide, and bortezomib in the twenty-first century has substantially changed the management of MM and markedly improved OS [12]. In particular, a significant 5-year OS improvement was seen in patients aged 45–64 years, while it was lower in patients aged 65–74, and absent in patients over 75 years of age [13]. The elderly population is highly heterogeneous and the well-known biologic and genetic prognostic factors, as well as age per se, are insufficient to explain this OS difference [14–17].

Indeed, aging is a complex process characterized by a gradual, progressive decrease in physiological reserve, changes in body composition, and clinically significant reductions in organ functions. It is commonly associated with the presence of comorbidity and an increased risk of developing disability, with physical and cognitive decline [18, 19], and frailty. Frailty is a state of increased vulnerability, with cumulative deficits in several physiological systems [11], which may result in a diminished resistance to stressors, such as MM and its treatment, negatively affecting patients' quality of life (QoL), treatment efficacy, and tolerability.

In MM, the term "frail" often improperly refers to a person >75 years, which sometimes leads to an inadequate under-treatment of patients based only on age. Of note, registrational trials are performed in selected patients, whereas frail patients usually do not meet eligibility criteria and thus are usually underrepresented in clinical studies [20].

Definition of Frailty

Older people are at high risk of cardio- and cerebrovascular diseases, type 2 diabetes, dementia, and cancer [21]. In particular, persons over 65 account for about 60% of newly diagnosed malignancies and about 70% of all cancer deaths. Elderly patients with cancer and comorbidities are also more likely to develop frailty, as well as physical and cognitive decline, with negative impact on lifestyle and treatment efficacy.

Indeed, the aging process is associated with a gradual, progressive decrease in physiological reserve, with changes in body composition and clinically significant reductions in renal function, gastric function, hepatic mass and blood flow, bone marrow status, and cardiovascular function [18, 22–24]. These changes affect the pharmacokinetics and pharmacodynamics of drugs, altering clinical efficacy and potentially increasing toxicity. Therefore, age-related organ function and metabolic changes can contribute to the poor tolerability of cancer treatments seen in elderly patients that can cause an increase in treatment-related adverse events. In these patients inadequate treatments can lead to excessive toxicities, unacceptable, unfavorable QoL, and increased healthcare costs.

In geriatric medicine, three terms are commonly used to characterize vulnerable adults: *frailty; comorbidity* (or multiple chronic conditions); and *disability* [25]. These are distinct clinical entities that occur individually and commonly in elderly patients. Moreover, they are interrelated and have a cumulative effect on health and prognosis of elderly patients. Of note, the use of score tables established in geriatric medicine provides additional information to performance status: in fact, 9–38% of elderly patients with good performance status (<2) were partially or fully dependent on others to carry out ordinary activities, such as household tasks and personal care [26, 27].

Frailty is a state of increased vulnerability to poor resolution of homoeostasis after a stressor event [28, 29]. It arises from cumulative deficits in several physiological systems and results in a diminished resistance to stressors. This is a condition more frequent in older individuals, as around 25% to 50% of people over 85 years of age are estimated to be frail. As yet, there is no consensus on the definition and assessment of frailty. The original definition of frailty focused on physical weakness and wasting, but many other definitions and criteria have been postulated, incorporating different aspects of aging that contribute to diminish physiological reserves. A phenotype of the clinically frail elderly adult was recently defined, based on the presence of a critical mass of ≥3 core elements of frailty: weakness; poor endurance; weight loss; low physical activity; and slow gait speed. *Comorbidity* is the concurrent presence of two or more medically diagnosed diseases in the same individual; it is associated with polymedication and increased risk of drug interactions [26]. Comorbidity is a significant concern among the elderly and 88% of the population aged over 65 years have at least one chronic condition. Many prognostic indices for the elderly that incorporate age and/or comorbidity are available. The Charlson comorbidity index is the one most frequently used in cancer patients [30, 31]. The Charlson index is a summary measure of 19 comorbid conditions weighted 1–6 corresponding to disease severity. This gives a total score ranging from 0 to 37. It can be

adapted to account for increasing age, adding 1 point to the score for each decade over the age of 50 years. With this index, the relative risk of death that can be attributed to an increase of 1 point in the comorbidity score is equivalent to an additional decade of age. With aging, the incidence of comorbid conditions increases markedly, largely because the frequency of individual chronic conditions rises with age. As a result, 35% of men and 45% of women aged 60–69 years in the United States have ≥2 comorbid conditions; this percentage increases dramatically to 53% of men and 70% of women by age 80 years [32].

Disability (which can include both physical and mental impairments or limitations) can be defined as difficulty or dependency in carrying out activities essential to independent living, including both essential personal care and household tasks, and activities that are important to maintain an individual's quality of life [33, 34]. Physical disability is common among elderly adults and is more common in women than men. The major causes of physical disability in the elderly are chronic diseases, such as cardiovascular disease, stroke, and arthritis, highlighting the interrelationship between disability and comorbidity [35]. The incidence of disability rises steadily with age among people aged ≥65 years [34]. Of community-dwelling adults, 20–30% of those aged >70 years report some disability in mobility, tasks essential to household management (e.g., shopping, meal preparation, managing money), and basic self-care tasks (e.g., washing, dressing, eating). Disability is associated with a higher probability of hospitalization and risk of mortality [36]. In oncologic-hematologic patients, the performance status generally describes the overall fitness of patients by a systematic scoring system (KPS or Eastern Cooperative Oncology Group [ECOG]), but the use of scales established in geriatric medicine to screen for disability in self-care tasks (activities of daily living, ADL) and tasks of household management (instrumental activities of daily living, IADL) provides additional information to simpler performance status. Several organizations recommend that persons older than 70 years be screened with ADL and IADL scales on an annual basis [37].

Geriatric Assessment

Determining the optimal treatment for older patients according to their clinical characteristics and fitness is the modern challenge in geriatric onco-hematology. A personalized treatment, tailored not only to disease-specific parameters but also to patient's health status is fundamental.

To date, chronological age and performance status are the most frequently used instruments to stratify patients according to their fitness and consequently select therapy. However, geriatric impairments are highly prevalent in elderly patients with hematological malignancies (even in those with good performance status), they may not be easily detected, and may impact on the patient's ability to complete treatment [38, 39].

A comprehensive geriatric assessment (CGA)—commonly used in geriatric oncology—is a systematic procedure to objectively evaluate the health status of older people, focusing on somatic, functional, and psychosocial domains [40]. The CGA is a highly sensitive and specific tool, and it is more objective and reliable than clinical judgment. Alternatively, a brief assessment with screening tools may be adopted at diagnosis in order to help clinicians identify patients who need a deeper evaluation through a CGA [41, 42].

The International Society of Geriatric Oncology task force recommended that a CGA be implemented for older cancer patients [41]. A growing interest in the geriatric assessment has recently emerged to evaluate older patients with hematological malignancies. In particular, a geriatric assessment showed to predict survival in older AML patients [43]. A simplified geriatric assessment proved to be more accurate than the physicians' judgment to predict response, progression-free survival (PFS), and OS in older patients receiving chemo-immunotherapy in non-Hodgkin lymphomas [39].

Geriatric Assessment in Multiple Myeloma

Recently the International Myeloma Working group has conducted a pooled analysis of 869 individual newly diagnosed elderly patient data from three prospective trials and proposed a frailty score for the measurement of frailty in elderly myeloma patients [44]. At diagnosis, a geriatric assessment was performed and included three tools: the ADL and the IADL scales to assess self-care activities, tasks of household management, and independence status; and the CCI to evaluate number and severity of comorbidities (Tables 27.1 and 27.2) [31, 45]. An additive scoring system (range 0–5) based on these three tools and on patients' age was developed, and three groups of patients were identified: fit (score = 0, 39%), intermediate (score = 1, 31%), and frail (score ≥ 2, 30%). The 3-year OS was 84% in fit, 76% in intermediate (hazard ratio [HR], 1.61; P = 0.042), and 57% in frail (HR, 3.57; $P < 0.001$) patients. The cumulative incidence of grade ≥3 non-hematologic adverse events at 12 months was 22.2% in fit, 26.4% in intermediate (HR, 1.23; P = 0.217), and

34.0% in frail (HR, 1.74; $P < 0.001$) patients. The cumulative incidence of treatment discontinuation at 12 months was 16.5% in fit, 20.8% in intermediate (HR, 1.41; $P = 0.052$), and 31.2% in frail (HR, 2.21; $P < 0.001$) patients. Frailty was found to be associated with an increased risk of death, progression, non-hematologic toxicities, and treatment discontinuation, regardless of International Staging System stage, chromosome abnormalities, and treatment.

Because performing a geriatric assessment can be manpower and time consuming, a simple computer application was also created to support clinicians.

This geriatric score was also validated in the phase 3 FIRST trial [46, 47]. Patients were categorized into three severity groups as described by a proxy algorithm based on the IMWG frailty scale and including age, EQ-5D: Self Care score, EQ-5D: Usual Activities score, and CCI index. Of 1517 patients, 17% were classified as fit, 30% as intermediate, and 54% as frail. Similar breakdowns were observed across treatment arms. Frail patients were older and had higher International Staging System stage, higher Eastern Cooperative Oncology Group performance status scores, higher lactate dehydrogenase levels, and worse renal function than fit or intermediate patients. Of note, fit patients had a significantly longer OS: fit vs. intermediate (hazard ratio [HR] 0.66; $P = 0.004$), fit vs. frail (HR 0.42; $P < 0.0001$), and intermediate vs. frail (HR 0.62; $P < 0.0001$). This analysis of the FIRST trial population using a proxy of the IMWG frailty scale demonstrated predictive clinical outcomes in patients with newly diagnosed MM similar to the original scale. The majority of patients fell into the frail category, demonstrating that FIRST studied a high risk population with poor outcomes and unmet need.

Table 27.1 Scores used to perform a geriatric assessment: Activities of daily living (ADL), instrumental activities of daily living (IADL), and Charlson comorbidity index (CCI)

ADL			IADL		CCI	
Activity	Independent	Dependent	Activity	Related score	Comorbidity	Related score
BATHING Patient bathes completely autonomously or needs help in bathing only a single part of the body such as the back, genital area, or disabled extremity	1	0	**Ability to use telephone**		Myocardial infarct	1
			Patient can operate telephone on own initiative; looks up and dials numbers	1	Congestive heart failure	1
			Patient can dial a few well-known numbers	1	Peripheral vascular disease	1
			Patient answers telephone, but does not dial	1		
			Patient does not use telephone at all	0	Cerebrovascular disease	1
					Dementia	1
DRESSING Patient gets clothes from closets and drawers and puts on clothes. Patient my need help for tying shoes	1	0	**Shopping**		Chronic pulmonary disease	1
			Patient takes care of all shopping needs independently	1	Connective tissue disease	1
			Patient can do the shopping independently for small purchases	0	Mild liver disease	1
			They need to be accompanied on any shopping trip	0	Diabetes	1
			Completely unable to shop	0	Ulcer	1
TOILETING Patient goes to toilet, gets on and off, arranges clothes, cleans genital area without help (may use cane or walker for support and bedpan/urinal at night)	1	0	**Food preparation**		Diabetes with end-organ damage	2
			Patient plans, prepares, and serves adequate meals independently	1	Ictus	2
			They can prepare adequate meals if supplied with ingredients	0	Moderate-to-severe renal failure	2
			Patient heats and serves prepared meals or prepares meals but does not maintain adequate diet	0	Nonmetastatic solid tumor	2
			They need to have meals prepared and served	0	Leukemia	2

Table 27.1 (continued)

ADL		
Activity	Independent	Dependent
TRANSFERRING Patient can move in and out of bed or chair unassisted. Mechanical transferring aides are acceptable	1	0
CONTINENCE Patient exercises complete self-control over urination and defecation	1	0
FEEDING Patient gets food from plate into mouth without help. Preparation of food may be done by another person	1	0

IADL	
Activity	Related score
Housekeeping	
Patient maintains house alone with occasion assistance (heavy work)	1
They can perform light daily tasks such as dishwashing, bed making	1
They can perform light daily tasks, but cannot maintain acceptable level of cleanliness	1
Patient needs help with all home maintenance tasks	1
Patient does not participate in any housekeeping tasks	0
Laundry	
They can do personal laundry completely	1
Patient launders small items, rinses socks, stockings, etc.	1
All laundry must be done by others	0
Mode of transportation	
They can travel independently on public transportation or drives own car	1
Patient arranges own travel via taxi, but does not otherwise use public transportation	1
Patient travels on public transportation when assisted or accompanied by another	1
Travel limited to taxi or automobile with assistance of another	0
They can not travel at all	0
Responsibility for own medications	
Patient is responsible for taking medication in correct dosages at correct time	1
Patient takes responsibility if medication is prepared in advance in separate dosages	0
They are not capable of dispensing own medication	0
Ability to handle finances	
Patient manages financial matters independently (budgets, writes checks, pays rent and bills, goes to bank); collects and keeps track of income	1
Patient manages day-to-day purchases, but needs help with banking, major purchases, etc.	1
Incapable of handling money	0

CCI	
Comorbidity	Related score
Lymphoma, MM	2
Moderate-to-severe liver disease	3
Metastatic solid tumor	6
AIDS	6

ADL Activity of daily living, *IADL* instrumental activity of daily living, *CCI* Charlson comorbidity index

Table 27.2 Definition of frail patients with MM

	Frail patients can either be:		
Age	>80 years	76–80 years	≤75 years
Geriatric assessment	Independently of ADL, IADL, CCI	Plus at least one of the following: • ADL ≤ 4 • IADL ≤ 5 • CCI ≥ 2	Plus at least two of the following: • ADL ≤ 4 • IADL ≤ 5 • CCI ≥ 2

ADL Activity of daily living, *IADL* instrumental activity of daily living, *CCI* Charlson comorbidity index
The geriatric score can be calculated through the website http://www.myelomafrailtyscorecalculator.net/

Therapy at Diagnosis for Frail Patients

Start of Treatment

Particular attention is needed with frail patients and their comorbid conditions. Frail patients may present with CRAB-like symptoms that do not lead to actual organ dysfunction and do not require immediate anti-myeloma treatment, but only a close monitoring [3, 4, 48, 49]. Patients may have age-related osteopenia, or mild renal impairment due to hypertension or diabetes, or mild anemia secondary to iron or vitamin deficiency, renal failure, chronic inflammatory diseases, or concomitant dyserythropoietic/myelodysplastic syndrome [50]. Conversely, clear clinical manifestations of serious end-organ damage attributable to myeloma should be considered as CRAB, such as a progressive worsening of serum creatinine caused by light-chain cast nephropathy or a decrease in hemoglobin levels from baseline. Creatinine clearance should be considered with caution. The methods commonly used to estimate glomerular filtration rate have not been well validated at the extremes of age. In frail patients, a progressive renal impairment rather than a fixed concentration cutoff should be considered to confirm MM diagnosis.

According to the recently updated International Myeloma Working Group criteria, clonal bone marrow plasma cell percentage ≥60%, involved/uninvolved serum free light chain ratio ≥100, and >1 focal lesions on magnetic resonance imaging (MRI) studies are associated with near-inevitable development of CRAB features and should be included among the parameters for the diagnosis of MM [4]. Even if no data are present about frail patients, the assessment of these parameters may avoid serious organ damage that could inevitably worsen patients' condition.

The initial evaluation of MM frail patients includes the analysis of their medical history, physical examination, laboratory evaluation, bone marrow biopsy, and aspirate with conventional cytogenetics and fluorescence in situ hybridization for recurring chromosomal translocations and deletions/duplications seen in MM [11]. To assess bone disease, more complex investigations, such as MRI or positron emission tomography (PET), can be unnecessary on a routine basis in frail patients, and should be used only in selected cases, whereas skeletal survey and low-dose computed tomography scan are routine investigations.

Treatment Options

In the last decade, new effective treatments including novel agents thalidomide, bortezomib, and lenalidomide have replaced the former standard melphalan-prednisone [1]. Today, MPT and VMP are the standard treatments for elderly patients ineligible for ASCT [6, 7]. Recently, Rd continuously was shown to be more effective than MPT at diagnosis [46]. It should be noted that currently approved combinations were validated in studies that included selected elderly patients, and a geriatric evaluation was not performed [29].

The benefits obtained with new drug-based combinations were limited in older patients, mainly due to an increased treatment-related toxicity. Indeed, advanced age (≥75 years), occurrence of severe adverse events, and drug discontinuation predicted shorter survival in newly diagnosed MM patients treated with melphalan-prednisone alone or in combination with thalidomide and/or bortezomib [51, 52]. Therefore, avoiding treatment interruption and reducing the risk of side effects in the initial phase of therapy are fundamental, and low-dose intensity treatments are appropriate options for frail patients.

In patients with symptomatic myeloma, patient's age and a geriatric assessment should be considered to determine the most appropriate treatment. Based on the results of the geriatric assessment, patients can be stratified into fit patients suitable for full-dose therapy with three-drug combinations, or frail patients requiring dose-adjusted therapies. Treatment strategies for frail patients should have minimal cumulative toxicity and two-drug regimens showed similar efficacy and lower toxicity as compared to multidrug combinations [53].

A recent phase 3 trial compared the triplet melphalan-prednisone-lenalidomide (MPR) and cyclophosphamide-prednisone-lenalidomide versus the doublet Rd in newly diagnosed elderly MM. The three-drug alkylator-containing combinations were not superior to the two-drug combination Rd. In addition, grade ≥3 toxicity neutropenia was significantly higher with MPR (64%) than with Rd (25%; $P < 0.0001$) [54].

In another trial, the doublet bortezomib-dexamethasone (VD) was as effective as the triplets VMP and bortezomib-thalidomide-dexamethasone in elderly patients, and induced a lower rate of non-hematologic adverse events (22% compared with 33–37% with the three-drug combinations), and thus should be preferred in this patient population. Although

all bortezomib-containing regimens produced good outcomes, VTD and VMP did not appear to offer an advantage over VD in patients with myeloma treated in US community practice [53].

A phase 2 trial evaluated three low-dose intensity subcutaneous bortezomib-based treatments in patients aged 75 years or older with newly diagnosed multiple myeloma (MM) [55]. Patients received subcutaneous bortezomib and oral prednisone (VP) or plus cyclophosphamide (VCP) or VP plus melphalan (VMP), followed by bortezomib maintenance, and half of the patients were frail. Response rate was 64% with VP, 67% with VCP, and 86% with VMP, and very good partial response rates or better were 26%, 28.5% and 49%, respectively. Median PFS was 14.0, 15.2, and 17.1 months, and 2-year OS was 60%, 70%, and 76% in VP, VCP, and VMP, respectively. At least one grade ≥3 nonhematologic adverse event occurred in 22% of VP, 37% of VCP, and 33% of VMP patients; discontinuation rates for AEs were 12%, 14%, and 20%, and the 6-month rates of toxicity-related deaths were 4%, 4%, and 8%, respectively. Yet, toxicity was higher with VMP, suggesting that a two-drug combination followed by maintenance should be preferred in frail patients [55].

These data underline the importance of avoiding treatment interruption and reducing the risk of side effects during the initial phase of therapy; thus low-dose intensity treatments should be preferred for frail patients.

Maintenance therapy showed to be effective also in elderly MM patients [1, 56]. Continuous Rd improved PFS compared with fixed-duration Rd for 18 cycles (Rd18) and significantly prolonged PFS and OS compared with MPT [46]. Continuous Rd caused a modest increase in toxicity compared with Rd18, and most of the toxicity occurred within the first 18 months and decreased over time. The superiority of continuous Rd over MPT for both PFS and OS was evident also in patients >75 years. Although toxicities were more common in patients >75 years, there was no marked difference in the rates of adverse events with continuous Rd and Rd18 within this age subgroup. Furthermore, Rd continuous resulted in PFS and OS benefits compared with MPT for patients of all frailty levels [47].

On the contrary, melphalan–prednisone–lenalidomide followed by lenalidomide maintenance (MPR-R) compared to MPR or melphalan-prednisone followed by placebo improved PFS in patients 65–75 years and not in patients >75 years [57]. This can be due to the higher toxicity of MPR and the need for more frequent dose modifications in patients >75 years. Of note, the major PFS benefit was achieved with lenalidomide maintenance therapy. In a landmark analysis, lenalidomide maintenance reduced the rate of progression by 66% as compared with placebo, regardless of age.

A main goal in frail patients is to maintain an asymptomatic disease status because older age and comorbidities may compromise subsequent salvage therapies. The benefits associated with continuous therapy should be balanced against the toxicity due to prolonged drug exposure, and this is particularly important in frail patients, who are more susceptible to treatment-related toxicities. Maintenance is a valuable option in frail patients who respond slowly to treatment and who tolerate it well. However, in case of significant adverse effects, dose reductions or treatment interruption should be considered.

Complete response has become an achievable aim also in elderly patients, yet toxicity may cancel the benefits derived from such a response [58]. Nevertheless, in frail patients the aim of therapy should not focus on the depth of response, but rather on controlling symptoms, maintaining an independence status and preserving quality of life. Therefore, the achievement of a stable disease without symptoms related to myeloma is an acceptable goal and keeping the balance between disease control and toxicity is crucial.

Dosing and Schedule

Dosing, schedule, and route of administration play a major role in the safety profile of therapy in frail patients. Once-weekly bortezomib significantly reduced the incidence of grade 3–4 adverse events (35% vs. 51%) and the rate of discontinuation due to toxicity (17% vs. 23%) compared with the twice-weekly schedule, in particular halving the rate of peripheral neuropathy. To overcome the limitations of bortezomib (intravenous administration and hospitalization), subcutaneous bortezomib is a valuable and equally effective strategy.

Lenalidomide has the advantage of the oral administration, which is more appealing in a frail patient population, and is preferable in subjects with preexisting neuropathy [46]. In particular, lenalidomide plus low-dose dexamethasone was better tolerated than lenalidomide plus high-dose dexamethasone [59]. Still, bortezomib-dexamethasone can be a valid option in case of aggressive disease, which needs a rapid cytoreduction and symptom control, and in case of acute renal dysfunction [53, 55].

In frail patients lower initial doses can be administered to minimize toxicity; dose escalation may be considered in the subsequent cycles if treatment is optimally tolerated or in case of inadequate response. The initial dose of lenalidomide should not exceed 10–15 mg/day and it can be adjusted based on renal function and blood counts to avoid profound and prolonged myelosuppression. Prophylactic growth factors and antimicrobial, at fixed dose and timing, can be used to prevent myelosuppression and infections, and thus treatment discontinuation. A previous history of cardiovascular disease or thromboembolism does not preclude the use of lenalidomide, if an adequate thromboprophylaxis is associated [11].

Corticosteroids may increase blood pressure and fluid retention; therefore they should be reduced, particularly in patients with cardiac diseases. Other adverse effects include hyperglycemia, gastritis, mood swings, insomnia, and increased risk of opportunistic infections. The dose of dexamethasone should be reduced to as little as 10 mg once a week. Alternatively, prednisone 25 mg every other day is a valid option in this setting [11].

Subcutaneous weekly bortezomib is a valid alternative option; it does not need dose reductions, although thrombocytopenia could be a concern [60–62]. This is a relevant strategy to improve tolerability, reduce treatment discontinuation, and increase the chance of disease control in frail patients. Bortezomib can cause peripheral neuropathy, differently from novel proteasome inhibitors [63, 64]. In frail patients, weekly carfilzomib, oral ixazomib, and oprozomib may be implemented in the future.

Finally, in patients with severe impairment of cognitive function or social dependency, a palliative treatment may be considered. In these cases, reduced-dose corticosteroids or melphalan-prednisone or cyclophosphamide-prednisone can be used to relieve disease symptoms.

Management of Complications

Despite the benefits associated with novel agents thalidomide, lenalidomide, and bortezomib, treatment-related toxicities are a major concern in frail patients. Full drug doses are difficult to be tolerated and side effects are frequent. In a retrospective analysis of 1435 elderly patients enrolled in four European phase III trials including thalidomide and/or bortezomib, the risk of death was increased in patients >75 years (HR 1.44, 95% CI: 1.20–1.72; $P < 0.001$); in patients with renal failure (HR 2.02, 95% CI: 1.51–2.70; $P < 0.001$); in those who had grade 3–4 infections, cardiac, or gastrointestinal adverse events during treatment (HR 2.53, 95% CI: 1.75–3.64; $P < 0.001$); and in those who discontinued therapy due to adverse events (HR 1.67, 95% CI; 1.12–2.51; $P = 0.01$). This increased risk was detected in the first 6 months after occurrence of adverse events or drug discontinuation and decreased over time [65]. More intensive approaches, such as the combination of bortezomib thalidomide, negatively affected outcome. Age 75 years or over or renal failure at presentation, occurrence of infections, and cardiac or gastrointestinal adverse events negatively affected survival.

Because frail patients are more susceptible to treatment-related side effects and treatment interruption, appropriate supportive care and an early identification of toxicities are fundamental [11].

Cytopenia is typical in hematologic patients treated with chemotherapy. It involves one or more cellular lineages of the bone marrow, due to both marrow invasion by neoplastic cells and chemotoxicity. Supportive care is essential to maintain an adequate quality of life and to enable the patient to stay on treatment [66].

Anemia is characterized by a hemoglobin level inferior to 13.5 g/dL for men and 12.0 g/dL for women. In elderly cancer patients anemia may be due to bone marrow invasion and chemotoxicity, but there are also other causes, such as renal insufficiency; iron, copper, and vitamin deficiency (folic acid, B12, and vitamin D); hypogonadism; relative erythropoietin system impairment; and possibly an underlying myelodysplasia or exhaustion of the hematopoietic progenitor. Approximately 50% of patients with anemia had two or more interconnected causes. Blood transfusions are recommended with hemoglobin levels below 7–8 g/dL. Erythropoietin-stimulating agents can improve fatigue and quality of life, and treatment could be continued until hemoglobin levels rise to 12 mg/dL [67].

Patients with neutropenia are also at risk of infections following chemotherapy, based on the severity and duration of neutropenia. Antibacterial prophylaxis can prevent febrile episodes, clinically or microbiologically documented bacterial infections including bacteremias, and hospitalization of outpatients [68]. Antibiotic prophylaxis should be started promptly when neutropenia occurs. Prophylaxis with antifungal agents (usually azoles) is recommended in cases of prolonged neutropenia. All patients undergoing chemotherapy or novel agent-based therapy should receive trimethoprim-cotrimoxazole as a prophylactic agent against the opportunistic infection of *Pneumocystis jiroveci* pneumonia.

Febrile neutropenia is characterized by an oral temperature higher than 38.5 °C or two consecutive measurements with a temperature higher than 38.0 °C for 2 h and an absolute neutrophil count of less than 0.5×10^9/L or one expected to fall below 0.5×10^9/L.

Chemotherapy-induced febrile neutropenia is a major risk factor for infection-related morbidity and death, as well as a significant dose-limiting toxicity in cancer treatment. Prognosis is worse in patients with bacteremia, with mortality rates of 18% in Gram-negative and 5% in Gram-positive bacteremia [69]. In elderly patients at high risk of developing febrile neutropenia, prophylactic granulocyte colony-stimulating factor (G-CSF) should be adopted based on age, medical history, disease characteristics, and myelotoxicity of their chemotherapy regimen. In patients with febrile neutropenia, the choice of initial antibiotic should be based on the patient's infectious disease history, prior antibiotic usage, and epidemiologic data of the area where the patient lives.

In patients receiving proteasome inhibitors, antiviral prophylaxis for herpes zoster reactivation is needed; immunomodulatory agents require an appropriate risk-based thromboprophylaxis. When corticosteroids are administered, gastrointestinal prophylaxis should be used. Antibacterial prophylaxis is recommended in case of severe myelosuppression,

and growth factors should be used in patients experiencing neutropenia and anemia. In addition, a careful review of the patient's previous medications and attention to potential drug interactions are essential [11].

When an adverse event occurs in a frail patient, prompt action is required. Therapy should be stopped in case of grade 3–4 toxicity, and can be restarted at lower doses when toxicity decreases to at least grade 1 [1]. Lenalidomide dose may be decreased from 15 to 10 mg/day, or from 10 to 5 mg/day or, if required, to 5 mg every other day on days 1–21 every 4 weeks. Bortezomib may be reduced from 1.3 mg/m^2 weekly to 1.0 mg/m^2 once weekly or 0.7 mg/m^2 once weekly.

Conclusions

Treatment of elderly patients is particularly challenging today because of the complex phenomenon of aging. Aging is in fact associated with an increased incidence of tumors and of other comorbidities, and more than two-thirds of cancer diagnoses and of cancer deaths are reported among elderly people [21].

The presence of multiple diseases in elderly patients and the availability of newer and more targeted drugs require tailored treatments according to both the disease features and the patient's status. In this setting, cancer treatment decision should be based not only on age but a careful assessment of patients' frailty is crucial. To date there is no consensus about the definition and assessment of frailty, although recently the International Myeloma Working Group has proposed a valuable tool to better stratify patients according to age, CCI, ADL, and IADL. However, the most appropriate treatment in different subgroups of patients remains to be definitively determined and further studies are necessary.

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Newly Diagnosed Multiple Myeloma in Transplant-Eligible Patients

28

Rajshekhar Chakraborty and Morie A. Gertz

Introduction

Multiple myeloma (MM) is characterized by clonal proliferation of malignant plasma cells in the bone marrow. An estimated 30,280 new cases of MM will be diagnosed in the USA in 2017 [1]. The introduction of proteasome inhibitors (PIs) and immunomodulators (IMiDs) in conjunction with autologous stem cell transplantation (ASCT) has markedly improved the overall survival (OS) in MM, with the survival gap between MM patients and matched controls decreasing in the last decade [2]. The incorporation of ASCT into the frontline therapy of transplant-eligible (TE) patients in the late 1990s had shown a remarkable improvement in survival in younger myeloma patients between 2001 and 2005 [3]. Currently, PI and/or IMiD-based combinations are the standard of care for pretransplant induction therapy in TE newly diagnosed MM patients [4]. Furthermore, the addition of long-term maintenance therapy after ASCT has also led to improved progression-free and overall survival [5]. Hence, there has been a paradigm shift in the management of newly diagnosed MM, leading to the achievement of a deep and durable disease control and improved survival, with the current median OS being greater than 6 years [6].

Diagnosis and Risk Stratification

Traditionally, therapy for MM was initiated once patients developed signs of end-organ damage or *CRAB,* signifying *h*ypercalcemia, *r*enal failure, *a*nemia, and *b*one lesions. However, in 2014, the International Myeloma Working Group (IMWG) proposed an updated criteria for diagnosis of MM, so that early therapy can be initiated to prevent end-organ damage [7]. In addition to the traditional *CRAB* features as a requirement for diagnosis and initiation of therapy in MM, the new IMWG criteria added three biomarkers: bone marrow plasma cells (BMPCs) ≥60%, serum free light chain ratio (sFLCr; involved/uninvolved) ≥100, or ≥1 focal lesion on magnetic resonance imaging (MRI). Currently, the IMWG recommends initiating therapy in MM in the presence of either of the above-mentioned biomarkers even if the patient does not exhibit any of the *CRAB* features and is asymptomatic. The rationale behind early initiation of therapy in MM is twofold: firstly, there are currently multiple effective agents available for frontline therapy in MM, including PIs and IMiDs and, secondly, early therapy in asymptomatic high-risk patients could potentially reduce the risk of end-organ damage and prolong overall survival, as seen in patients with high-risk smoldering myeloma [8].

Once a diagnosis has been established, risk stratification should be performed using one of the prognostic models summarized in Table 28.1 [9–11], which are routinely used in clinical practice. In the revised International Staging System (rISS) for MM [10], chromosomal abnormalities by fluorescence in situ hybridization (FISH) and lactate dehydrogenase (LDH) have been incorporated into the ISS staging [12], which included albumin and β-2 microglobulin. Presence of deletion(17p), t(4;14), and t(14;16) by FISH has an independent negative prognostic impact on survival in MM [10]. The 5-year OS rate of patients with rISS stage I, II, and III are 82%, 62%, and 40%, respectively. Notably, the patient population in rISS staging received up-front novel agents in 95% and ASCT in 60% of cases. Other risk stratification tools like gene expression profiling [13] have also been used, but are not widely available.

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Table 28.1 Risk stratification systems in multiple myeloma

Prognostic model	Variables	Risk categories	Overall survival	Ref.
rISS	ISS staging, CA [deletion(17p), t(4;14), t(14;16)] and LDH	**rISS stage I:** ISS stage I, no high-risk CA and LDH ≤ UNL **rISS stage II**: All other combinations **rISS stage III:** ISS stage III *AND* high-risk CA or LDH > UNL	5-year OS rate: rISS-I: 82% rISS-II: 62% rISS-III: 40%	
mSMART	CA, GEP, and PCLI	**Standard risk**: All others including t(11;14) or t(6;14) on FISH **Intermediate risk**: t(4;14) on FISH, cytogenetic deletion 13, hypodiploidy or PCLI ≥3% **High risk**: Del(17p), t(14;16) or t(14;20) on FISH or high-risk signature on GEP	Median OS: Standard risk: 8–10 years Intermediate risk: 4–5 years High risk: 3 years	
IMWF	ISS staging and CA	**Low risk**: ISS stage I/II *AND* absence of t(4;14), deletion(17p) and +1q21 *AND* age <55 years **Standard risk**: Others **High risk**: ISS stage II/III *AND* t(4;14) or 17p13 deletion	Median OS: Low risk: 2 years Standard risk: 7 years High risk:>10 years	
IFM	LDH, ISS (III), and cytogenetics (FISH)	**Scores 0–3** (higher score indicating poor prognostic subgroup), with 1 point each for high LDH, ISS stage III, and high-risk CA [t(4;14) and/or del(17p)]	2-year OS rate: Score 0: 93% Score 1: 85% Score 2: 67% Score 3: 55%	

Optimal Induction Regimen for Transplant-Eligible Patients

The goal of induction therapy in MM is to rapidly reduce the tumor burden for minimizing the risk of end-organ damage with minimal toxicities. Furthermore, in TE patients, stem cell toxic drugs like alkylating agents should be avoided due to the risk of inadequate stem cell mobilization. Fortunately, with the introduction of PIs and IMiDs in the treatment armamentarium of MM, a wide variety of non-stem cell toxic induction regimens with high anti-myeloma activity are available in the current era. In TE patients, the combination of bortezomib and dexamethasone (VD) was shown to be superior to alkylating agent-based vincristine-adriamycin-dexamethasone (VAD) induction regimen in terms of response rate and progression-free survival (PFS) in the phase III IFM trial, with less toxicity [14]. Subsequently, a meta-analysis of three phase III RCTs on bortezomib- versus non-bortezomib-containing induction regimen prior to ASCT showed a superior PFS and OS with bortezomib-containing induction regimens [15], the comparator being alkylating agent in two and thalidomide-based induction regimen in one trial. These studies have paved the way for several combination regimens on a backbone of VD to be widely used as induction therapy in newly diagnosed TE-MM patients [4, 16, 17]. However, with a wide variety of available induction regimens, the optimal choice for frontline induction therapy is becoming increasingly complex. The 2017 NCCN guideline recommends using a three-drug over two-drug regimen for frontline therapy in MM based on the evidence of improved response rates and survival [4]. A study on serial genomic analysis of MM has shown that the tumor can exhibit either genomic stability, linear evolution, or clonal heterogeneity with shifting clonal predominance over time [18]. Furthermore, the genome of patients with standard-risk cytogenetics showed minimal changes over time, unlike those with high-risk cytogenetics which was characterized by clonal evolution. This study provides the biological rationale for preferentially using three-drug over two-drug regimens since a suboptimal frontline therapy especially in high-risk patients may fail to eradicate the aggressive clones and lead to future relapse due to clonal evolution. In patients with high-risk cytogenetic abnormalities like deletion (17p), three-drug regimens incorporating a PI and IMiD like bortezomib-lenalidomide-dexamethasone (VRD) or carfilzomib-lenalidomide-dexamethasone (KRD) should be used for induction therapy. Bortezomib-based regimens should be used in those presenting with renal insufficiency at diagnosis [19]. Medical comorbidities, including preexisting peripheral neuropathy should also be taken into consideration while choosing the induction regimen. Intensive induction therapy with bortezomib and/or alkylating agents followed by ASCT is recommended for patients presenting with primary plasma cell leukemia (PCL) [20].

The commonly used induction regimens along with the evidence supporting their use are summarized below and the important phase II and III clinical trials on induction regimens in TE patients are shown in Table 28.2 (three-drug combinations) and Table 28.3 (two-drug combinations).

Table 28.2 Induction regimens with three-drug combinations in transplant-eligible patients

Author	Phase	No.	Regimen used	Response rate		Long-term outcomes		Ref.
				Post-induction	Posttransplant	PFS	OS	
VRD (bortezomib-lenalidomide-dexamethasone)								
Kumar et al.	II	VRD:42 VCD:33 VCRD: 48	***VRD***: V 1.3 mg/m² days 1, 4, 8 and 11; R 25 mg days 1–14; D 40 mg days 1, 8, and 15 (21-day cycle) ***VCD***: V and D as above, C 500 mg/m² days 1 and 8 (21-day cycle) ***VCRD***: V, D, R, and C as above (21-day cycle) *Schema:* 8 cycles of induction [ASCT any time after 4 cycles in TE patients] → V*m* × 4 cycles	≥VGPR rate: VRD: 51% VCD: 41% VCRD: 58%	NR	1-year PFS rate: VRD: 83% VCD: 93% VCRD: 86%	1-year OS rate: VRD: 100% VCD: 100% VCRD: 92%	
Roussel et al.	II	31	***VRD***: V 1.3 mg/m² days 1, 4, 8 and 11; R 25 mg days 1–14; D 40 mg days 1, 8, and 15 (21-day cycle) *Schema:* VRD × 3 cycles → ASCT (Mel200) → VRD × 2 cycles → R*m* × 1 year	≥VGPR rate: 58%	≥VGPR rate: 70%	3-year PFS rate: 77%	3-year OS rate: 100%	
VTD (bortezomib-thalidomide-dexamethasone)								
Cavo et al.	III	480	***VTD:*** V 1.3 mg/m² days 1, 4, 8, and 11; T 100 mg days 1–14 and 200 mg days 15–21; D 40 mg on 8 of first 12 days (21-day cycle) ***TD:*** T and D as above (21-day cycle) VTD arm: VTD × 3 cycles → ASCT × 2 (Mel200) → VTD*c* × 2 cycles TD arm: TD × 3 cycles → ASCT × 2 (Mel200) → TD*c* × 2 cycles	≥VGPR rate: VTD: 62% Td: 28% ($P < 0.001$)	≥VGPR rate: VTD: 82% Td: 64% ($P < 0.001$)	3-year PFS rate: VTD: 68% Td: 56% ($P = 0.006$)	3-year OS rate: VTD: 86% Td: 84% ($P = 0.30$)	
Rosiñol et al.	III	386	***VTD:*** V 1.3 mg/m² days 1, 4, 8 and 11; T 200 mg days 1–28; D 40 mg on days 1–4 and 9–12 (28-day cycle) ***TD:*** T and D as above (28-day cycle) ***QT-V:*** Alkylating agent-based chemotherapy; V as above (5-week cycle) VTD arm: VTD × 6 cycles → ASCT (Mel 200) → IFN*m or* T*m or* VT*m* TD arm: VTD arm: VTD × 6 cycles → ASCT (Mel 200) → IFN*m or* T*m or* VT*m*	CR rate: VTD: 35% Td: 14% ≥VGPR rate: VTD: 60% Td: 29% ($P < 0.001$)	CR rate: VTD: 46% Td: 24% ($P = 0.004$)	Median PFS: VTD: 56 months TD: 28 months ($P = 0.01$)	4-year OS rate: VTD: 74% Td: 65% (P = NS)	
Moreau et al.	III	340	***VTD:*** V 1.3 mg/m² days 1, 4, 8, and 11; T 100 mg days 1–21; D 40 mg on days 1–4 and 9–12 (21-day cycle) ***VCD:*** V and D as above; C 500 mg/m² on days 1, 8, and 15 (21-day cycle) VTD arm: VTD × 4 cycles → ASCT ± *c/m* VCD arm: VTD × 4 cycles → ASCT ± *c/m*	≥VGPR rate: VTD: 66% VCD: 56% ($P = 0.05$)	NR	NR	NR	
VCD (bortezomib-cyclophosphamide-dexamethasone)								
Reeder et al.	II	33	***VCD***: V 1.3 mg/m² on days 1, 4, 8, and 11; D 40 mg on days 1–4, 9–12, and 17–20; C 300 mg/m² days 1, 8, 15, and 22 (28-day cycle) *Schema:* VCD × 4 cycles → ASCT	≥PR rate: 88% ≥VGPR rate: 61%	NR	NR	NR	
Einsele et al.	II/III	300	***VCD:*** V 1.3 mg/m² on days 1, 4, 8, and 11; C 900 mg/m² day 1; D 40 mg on days 1–2, 4–5, 8–9, and 11–12 (21-day cycle) *Schema:* VCD × 3 cycles → ASCT (Mel 200)	≥PR rate: 74% ≥CR rate: 10%	NR	NR	NR	

(continued)

Table 28.2 (continued)

Author	Phase	No.	Regimen used	Response rate		Long-term outcomes		Ref.
				Post-induction	Posttransplant	PFS	OS	
Mai et al.	III	504	***VCD***: V 1.3 mg/m² daily on days 1, 4, 8, and 11; C 900 mg/m² day 1; D 40 mg on days 1–2, 4–5, 8–9, and 11–12 (21-day cycle) ***PAD:*** P as above; A 9 mg/m² on days 1–4; D 20 mg on days 1–4, 9–12, and 17–20 (28-day cycle) VCD arm: VCD × 3 cycles → ASCT (Mel 200) × 1 or 2 → R*m* × 2 years *or* until CR PAD arm: PAD × 3 cycles → ASCT (Mel 200) × 1 or 2 → R*m* × 2 years *or* until CR	≥VGPR rate: VCD: 37% PAD: 34.3%	NR	NR	NR	
Bortezomib-doxorubicin-dexamethasone (PAD)								
Sonneveld et al.	III	827	***VAD***: V 0.4 mg daily on days 1–4; A 9 mg/m² daily on days 1–4; D 40 mg daily on days 1–4, 9–12, and 17–20 (28-day cycle) ***PAD:*** P 1.3 mg/m² daily on days 1, 4, 8, and 11; A and D as above (28-day cycle) VAD arm: VAD × 3 cycles → ASCT (Mel 200) × 1 or 2 → T*m* × 2 years PAD arm: PAD × 3 cycles → ASCT (Mel 200) × 1 or 2 → V*m* × 2 years	≥VGPR rate: VAD: 14% PAD: 42%	≥VGPR rate: VAD: 36% PAD: 62%	Median PFS (month): VAD: 28 PAD: 35 ($P = 0.002$)	5-year OS rate: VAD: 55% PAD: 61% ($P = 0.07$)	
Carfilzomib-lenalidomide-dexamethasone (KRD)								
Jakubowiak et al.	I/II	53	***KRD:*** K 20, 27 or 36 mg/m² on days, 1, 2, 8, 9, 15, 16 and days 1, 2, 8, 9 after cycle 8; R 25 mg daily on days 1–21; D 40 mg in cycles 1–4 and 20 mg in cycles 5+ on days 1, 8, 15 and 22 (28-day cycle) *Schema:* KRD × 4 cycles → SCC and ASCT (for TE patients) or 4 more cycles for non-TE patients → KRD*m* cycles 9–24	≥VGPR rate: 81% ≥nCR rate: 62% sCR rate: 42%	NR	PFS rates: 1-year: 97% 2-year: 92%	NR	
Korde et al.	II	41	***KRD:*** K 20/36 mg/m² on days, 1, 2, 8, 9, 15, 16; R 25 mg daily on days 1–21; D 20/10 mg on days 1, 2, 8, 9, 15, 16, 22, and 23 (28-day cycle) *Schema:* KRD × 4 cycles → SCC → KRD × 4 cycles → R × 24 cycles for patients ≥SD	≥VGPR rate: 89% ≥nCR rate: 63% sCR rate: 41%	NR	NR	NR	
Gay et al.	II	281						
Ixazomib-lenalidomide-dexamethasone (IRD)								
Kumar et al.	I/II	65	***IRD:*** I 1.68–3.95 mg/m² on days, 1, 8 and 15; R 25 mg daily on days 1–21; D 40 mg weekly (28-day cycle) *Schema:* IRD × 12–28 cycles (SCC after 4 cycles in TE-patients) → I*m*	≥VGPR rate: 58% ≥CR rate: 27%	NR	1-year PFS rate: 88%	1-year OS rate: 94%	

Table 28.3 Induction regimens with two-drug combinations in transplant-eligible patients

Author	Phase	No	Regimen used	Response rate		Long-term outcomes		Ref.
				Post-induction	Posttransplant	PFS	OS	
VD (bortezomib-dexamethasone)								
Harrousseau et al.	III	482	***VD:*** V 1.3 mg/m^2 on days 1, 4, 8, and 11; D 40 mg days 1–4 (all cycles) and days 9–12 (cycles 1–2) [21-day cycles] ***VAD:*** Vincristine 0.4 mg/day and A 9 mg/m^2/day on days 1–4; D 40 mg daily days 1–4 (all cycles) and days 9–12 and 17–20 (cycles 1–2) [28-day cycles] VD or VAD × 4 cycles → ASCT (Mel200) × 1 or 2 → L*c* × 2 months → L*m* or placebo per IFM 2005-02 protocol	≥VGPR rate: VD: 54 VAD: 37 ($P < 0.001$)	NR	Median PFS: VD: 36 VAD: 30 ($P = 0.064$)	3-year OS rates: VD: 81.4% VAD: 77.4% ($P = 0.508$)	
Moreau et al.	III	199	***VD:*** V 1.3 mg/m^2 on days 1, 4, 8, and 11; D 40 mg days 1–4 (all cycles) and days 9–12 (cycles 1–2) [21-day cycles] ***rVTD:*** V 1 mg/m^2 on days 1, 4, 8, and 11; T 100 mg daily; D as above (21-day cycles) VD or rVTD × 4 cycles → ASCT (Mel200)	≥VGPR rate: VD: 49 rVTD: 36 ($P = 0.05$)	≥VGPR rate: VD: 74 rVTD: 58 ($P = 0.02$)	Median PFS: VD: 30 months rVTD: 26 months ($P = 0.22$)	No difference in OS between the two groups	
TD (thalidomide-dexamethasone)								
Rajkumar et al.	III	470	***TD:*** T 50 mg daily days 1–14, 100 mg daily days 15–28 and 200 mg from day 1 of cycle 2 (28-day cycle) ***D***: D 40 mg days 1–4, 9–12, 17–20 in cycles 1–4 and days 1–4 from cycle 5 onwards *Schema:* TD or D until progression	ORR: TD: 63 D: 46 ($P < 0.001$) ≥VGPR rate: TD: 44 D: 16 ($P < 0.001$)	NR	Median PFS: TD: 14.9 months D: 6.5 months	No significant difference in the two arms	
RD (lenalidomide-dexamethasone)								
Zonder et al.	III	198	***RD:*** R 25 mg daily in 28 of 35 days for induction and 12 of 28 days for maintenance; D 40 mg days 1–4, 9–12, and 17–20 for induction and days 1–4, 15–18 for maintenance (35-day cycles) ***D***: D as above *Schema:* RD or D × 3 cycles → R*m or Dm*	CR rate: RD: 22% D: 4% ($P = 0.001$)	NR	1-year PFS rate: RD: 77% D: 55% ($P = 0.002$)	1-year OS rate: RD: 93% D: 91% (P = NS)	
Rajkumar et al.	III	445	***RD:*** R 25 mg daily days 1–21; D 40 mg days 1–4, 9–12, 17–20 (28-day cycle) ***RlowD:*** R as above; lowD: D 40 mg on days 1, 8, 15, and 22 (28-day cycle) *Schema:* RD or RlowD for 4 cycles until ASCT or until progression	≥PR rate: RD: 79% RlowD: 68% ($P = 0.008$)	NR	Median PFS: RD: 19 months RlowD: 25 months ($P = 0.026$)	1-year OS rate: RD: 87% RlowD: 96% ($P = 0.0002$)	

Three-Drug Combinations

Bortezomib-Lenalidomide-Dexamethasone (VRD)

VRD was initially evaluated as a frontline induction therapy in the phase I/II study by Richardson et al. [21]. The regimen was shown to be highly effective with an unprecedented post-induction partial response (PR) rate of 100% and a VGPR rate of 67%. The most common grade 2/3 AEs were sensory neuropathy and fatigue and grade 3/4 hematologic toxicities included thrombocytopenia, neutropenia, and lymphopenia. The 18-month PFS and OS rates were 75% and 97%, respectively, at a median follow-up of 21 months. Subsequently, a phase II study by the French group on VRD induction followed by frontline ASCT in newly diagnosed MM patients demonstrated a post-induction VGPR rate of 58% [22]. Notably, 16% of patients in this trial achieved post-induction MRD negativity by flow cytometry at a sensitivity level of 10^{-4}. The 3-year PFS and OS rates were 77% and 100%, respectively, at a median follow-up of 39 months. No treatment-related mortality was reported. Discontinuation due to AEs was noted in only 3% of patients during induction or consolidation phase. The most common grade 3/4 adverse events (AEs) were neutropenia and thrombocytopenia. The randomized phase II EVOLUTION study compared VRD with bortezomib-cyclophosphamide-dexamethasone (VCD) and VRCD in newly diagnosed MM patients, majority of whom were TE [23]. There was no significant difference in the incidence of post-induction VGPR and 1-year PFS rate in the either arms. AEs leading to discontinuation of therapy were seen in 19% of patients in the VRD and 12% in the VCD arm.

Currently, VRD remains the preferred induction regimen in both TE and non-TE MM patients. Although there is no data on OS benefit with VRD in TE patients, it has been shown to have a superior OS over RD in non-TE patients in the SWOG S0777 trial [24].

Bortezomib-Thalidomide-Dexamethasone (VTD)

VTD was found to be superior to TD as a pretransplant induction regimen in the phase III GIMEMA trial [25]. In this trial, 480 patients were randomized to either VTD or TD induction and consolidation therapy in the setting of tandem ASCT. The post-induction VGPR rates in VTD and TD arms were 62% and 28%, respectively ($P < 0.001$). Patients in the VTD arm had a significantly superior 3-year PFS rate compare to those receiving TD (68% vs. 56%, respectively; $P = 0.006$). However, there was no significant difference in the 3-year OS rates at a median follow-up of 36 months. Of note, VTD was shown to abrogate the negative prognostic impact of t(4; 14) cytogenetic abnormality. The incidence of grade 3/4 AEs was 56% in the VTD arm and 33% in the TD arm, with no significant difference in treatment-related mortality. Notably, the incidence of grade 3/4 peripheral neuropathy (PN) was higher in the VTD (10%) compared to TD arm (2%). However, treatment discontinuation in the VTD arm was seen in only 9% of patients developing PN, with 78% experiencing improvement or resolution of symptoms over time.

Another phase III trial by the PETHEMA/GEM group randomized 386 TE patients to receive either VTD, TD, or multiple alkylating agent-based chemotherapy with bortezomib, followed by ASCT and maintenance therapy [26]. The post-induction complete response (CR) rate was 35% in the VTD arm, compared to 14% in the TD arm ($P = 0.001$). In patients with extramedullary soft-tissue plasmacytomas, the post-induction CR rates in VTD and TD arms were 42% and 14%, respectively ($P = 0.02$). The incidence of grade 2–4 PN with VTD was 60% compared to 13% with TD. Dose reduction of bortezomib due to PN was needed in 25% of patients in the VTD arm and 2% had to discontinue therapy. Although patients in the VTD arm had a superior PFS compared to TD (median, 56 vs. 28 months; $P = 0.01$), there was no significant difference in the 4-year OS rate at a median follow-up of 35 months. Patients with high-risk cytogenetics had an inferior PFS and OS irrespective of the treatment arm.

VTD has also been compared with VCD as an induction regimen prior to ASCT in a phase III randomized trial by the IFM group [27]. The post-induction VGPR rate was higher with VTD compared to VCD (66% vs. 56%, respectively; $P = 0.05$). The posttransplant response and survival were not reported in this trial. The incidence of grade 2–4 PN was higher in the VTD compared to VCD arm and grade 3/4 hematologic toxicity was higher in the VCD arm.

VTD remains an active induction regimen in TE patients, with the potential limitation being high rates of PN. Notably, it does not need any dose modification in patients with renal dysfunction and is less expensive compared to other novel agent-based triplet regimens like VRD or KRD.

Bortezomib-Cyclophosphamide-Dexamethasone (VCD)

A phase II trial of VCD induction in newly diagnosed TE-MM patients showed a post-induction PR rate of 88%, with a VGPR rate of 61% [28]. Common grade 3/4 toxicities included hematologic toxicity, hyperglycemia, diarrhea, neuropathy, and thrombosis. Another phase II trial comparing once- versus twice-weekly bortezomib in VCD induction found similar VGPR rates of 60% and 61%, respectively in the two cohorts [29]. However, grade 3/4 AEs, including PN, were lower in the once-weekly compared to the twice-weekly cohort, favoring the use of once-weekly bortezomib with similar efficacy and improved tolerability. Long-term follow-up of VCD induction therapy followed by ASCT has shown 5-year PFS and OS rates of 42% and 70%, respectively [30]. A phase II/III German study on VCD induction

in 300 TE patients showed 84% achieving a PR or better after induction, with 10% achieving a CR [31]. Serious AEs were observed in 26% of patients, with grade 3 PN in only 2.3%. Furthermore, VCD was shown to abrogate the negative prognostic impact of t(4; 14) abnormality. The phase II EVOLUTION study showed no significant difference in the post-induction response and 1-year PFS rates between VCD and VRD, as mentioned earlier [23]. The head-to-head comparison between VCD and VTD has shown higher post-induction VGPR rate in the latter but survival outcomes have not been reported thus far [27].

A phase III randomized trial comparing VCD with bortezomib-doxorubicin-dexamethasone (PAD) in 504 newly diagnosed TE-MM patients showed similar post-induction VGPR rates in the two arms (37% and 34% in VCD and PAD arms, respectively; *P* value for non-inferiority <0.001) [32]. The incidence of grade 2–4 neuropathy was higher in the PAD arm (15%) compared to the VCD arm (8%) [$P = 0.003$]. Similarly, severe AEs due to thromboembolic events were also higher in the PAD compared to the VCD arm (2.8% vs. 0.4%, respectively; $P = 0.04$). Stem cells were adequately collected for at least one transplant in both arms in close to 90% of patients. This study confirmed the non-inferiority in terms of efficacy and a superior safety profile of VCD compared to PAD as an induction regimen in TE-MM patients.

VCD is a highly effective induction regimen in TE patients. Further studies should aim at comparison of VCD with current standard-of-care regimens like VRD and VTD in terms of PFS and OS, as frontline VCD administration is cost effective and reserves the use of IMiDs later in the disease course [9].

Bortezomib-Doxorubicin-Dexamethasone (PAD)

PAD was compared head to head with VAD as a pretransplant induction regimen in the phase III HOVON/GMMG trial [33]. In this trial 827 patients were randomly assigned to receive three cycles of either PAD or VAD followed by a single or tandem ASCT. Patients in the PAD arm received 2 years of bortezomib maintenance after ASCT and those in the VAD arm received 2 years of thalidomide maintenance. The post-induction VGPR rates in the PAD and VAD arms were 42% and 14%, respectively ($P < 0.001$). Patients randomized to the PAD arm had a superior PFS compared to those in the VAD arm (median PFS, 35 vs. 28 months, respectively; $P = 0.002$) and the 5-year OS rates were 61% and 55%, respectively ($P = 0.07$), at a median follow-up of 41 months. Notably, in the subgroup of patients with deletion (17p) cytogenetic abnormality by FISH, the median PFS was 36 months in the PAD compared to 18 months in the VAD arm ($P < 0.001$) [34]. Similarly, the 3-year OS rates in the deletion (17p) subgroup was 83% and 36% in the PAD and VAD arms, respectively (HR 2.4 for VAD; $P < 0.001$). Furthermore, administration of bortezomib both before and after ASCT (as in the PAD arm) abrogated the negative prognostic impact of deletion (17p) [34]. Patients with renal failure also had a superior PFS and OS in the PAD arm [33]. Grade 2–4 PN was observed in 18% of patients receiving VAD and 40% receiving PAD. SCC was successful in close to 90% of patients in either arm.

Since patients with deletion (17p) have poor outcomes, administration of three-drug combinations including bortezomib or carfilzomib as pretransplant induction therapy and bortezomib maintenance after ASCT is recommended in this subgroup.

Carfilzomib-Lenalidomide-Dexamethasone (KRD)

Carfilzomib is a highly selective and irreversible next-generation PI with robust anti-myeloma activity [35, 36]. It has been shown to have a single-agent activity in relapsed/refractory (R/R) MM [37] and also in combination with RD in the phase III ASPIRE trial [38]. A phase I/II study of KRD in 53 patients with newly diagnosed MM showed an ORR of 98%, with 42% achieving a stringent CR (sCR) after induction [39]. At a median follow-up of 13 months, the 1- and 2-year PFS rates were 97% and 92%, respectively. Thirty-five patients in this trial underwent successful stem cell collection and seven eventually proceeded to ASCT. Common grade 3/4 AEs included hyperglycemia (23%), hypophosphatemia (25%), anemia (21%), thrombocytopenia (17%), and neutropenia (17%). PN was observed in 12% of patients, all being grades 1–2. There was no treatment-related mortality. Another phase II study of KRD induction followed by extended lenalidomide administration in 41 newly diagnosed TE and non-TE patients showed a post-induction VGPR rate of 89%, with a sCR rate of 41% [40]. Among 17 patients with at least a near CR (nCR) who underwent MRD testing at a sensitivity level of 10^{-5}, all were found to be MRD negative. Common non-hematologic grade 3/4 AEs included electrolyte disturbances (18%), abnormal liver function tests (13%), rash or pruritus (11%), fatigue (11%), cardiovascular toxicity (8%), and dyspnea (8%). The phase II FORTE trial has compared KRD and KCD (carfilzomib-cyclophosphamide-dexamethasone) in 281 TE-MM patients [41]. The post-induction rate of achieving VGPR or better response after four cycles of induction therapy was 74% with KRD and 61% with KCD ($P = 0.05$). The incidence of grade 3/4 AEs was higher in the KCD compared to KRD arm. On the other hand, grade 3/4 dermatological AEs and elevated liver function tests were common in the KRD arm. Stem cell mobilization was successful in more than 95% of patients in both arms.

KRD is emerging as a highly effective induction regimen in MM, with a high rate of sCR and MRD negativity post-induction. However, to the best of our knowledge, there is no phase III data on frontline therapy in TE-MM patients thus far. Although the incidence of PN is lesser with carfilzomib

compared to bortezomib, cardiotoxicity observed in the clinical trials of carfilzomib is concerning. A meta-analysis of phase I/II, II, and III clinical trials on carfilzomib including 2594 patients has shown the risk of all grade and grade 3/4 cardiotoxicity (including arrhythmias, systolic congestive heart failure, and acute coronary syndrome) to be 18% and 8%, respectively [42]. Furthermore, the cardiotoxicity was dose dependent, with patients receiving carfilzomib dose ≥45 mg/m^2 having a twofold higher incidence of grade ≥3 cardiac AEs, compared to those receiving <45 mg/m^2. Hence, caution should be exercised in patients at a high risk of cardiovascular complications.

Ixazomib-Lenalidomide-Dexamethasone (IRD)

The combination of oral PI ixazomib with RD has been shown to be safe and effective in 65 TE and non-TE patients in an open-label phase I/II trial [43]. Grade 3–4 AEs were observed in 63% of patients, including disorders of skin and subcutaneous tissue (17%), neutropenia (12%), thrombocytopenia (8%), and PN (6%). The post-induction ORR was 92%, including a VGPR rate of 58% and a CR rate of 27%. At a median follow-up of 14 months, the 1-year PFS and OS rates were 88% and 94%, respectively. SCC was attempted in 45% of patients after a median of four cycles of induction therapy. Although the activity of IRD seems promising in newly diagnosed MM, further data from phase II and III clinical trials on head-to-head comparison with currently used regimens is needed prior to its incorporation in the frontline therapy for TE patients.

Two-Drug Combinations

Bortezomib-Dexamethasone (VD)

VD was the first novel agent-based induction regimen which was shown to be superior to alkylating agent-based induction prior to ASCT in newly diagnosed MM [14]. In this phase III IFM trial, 482 patients were randomized to receive VD, VAD, VD followed by consolidation with multiple alkylating agents, or VAD followed by a similar consolidation. Subsequently, all patients would proceed to a single ASCT, with tandem ASCT reserved for those achieving less than a VGPR after the first ASCT. Post-induction VGPR rate was significantly higher with VD compared to VAD (54% vs. 37%, respectively; $P < 0.001$). Patients receiving VD had a superior PFS; however, they did not reach statistical significance (median PFS, 36 months with VD vs. 30 months with VAD; $P = 0.064$). Consolidation with alkylating agents did not improve post-induction response rate. Adequate SCC was achieved in more than 95% of patients in both arms. The incidence of grade 3/4 anemia, neutropenia, and thrombosis was significantly higher in the VAD arm, as was the incidence of toxicity-related deaths. On the other hand, grade 2/3 PN was significantly higher in the VD arm. Analysis of patients with high-risk FISH cytogenetics either enrolled or treated according to the above protocol showed that induction with VD abrogated the negative prognostic impact of t(4;14) but not deletion (17p) [44]. Another phase III trial by the French group randomized patients to receive VD or reduced-dose VTD (rVTD). The post-induction VGPR rate was significantly higher in the rVTD arm compared to the VD arm (49% vs. 36%; $P = 0.05$). However, there was no significant difference in PFS or OS. Notably, the incidence of grade 2–4 PN was 34% in the VD and 14% in the rVTD arm ($P = 0.001$).

Thalidomide-Dexamethasone (TD)

Thalidomide is a first-generation IMiD and is not widely used in the USA due to the availability of lenalidomide, which has a superior safety profile and a higher potency compared to thalidomide. The 2017 NCCN guidelines have removed TD as a recommended induction regimen in TE patients [4]. However, due to low cost, it still remains valuable in resource-limited settings, where there is a lack of access to bortezomib and lenalidomide.

TD was shown to be superior to D alone as an induction therapy in newly diagnosed MM in a phase III trial. The post-induction ORR was significantly higher with TD compared to D, which translated into a superior PFS (median PFS, 14.9 months with TD vs. 6.5 months with D; $P < 0.001$) [45]. However, grade 3/4 AEs, especially thromboembolic complications (18%) and PN (3.4%), were significantly higher with TD. Of note, routine thromboprophylaxis was not administered in this trial, which is currently a standard of care in patients receiving IMiDs. VTD induction therapy has been shown to have a superior PFS compared to TD in two phase III trials, as mentioned earlier [15, 25, 26].

Lenalidomide-Dexamethasone (RD)

Lenalidomide is a second-generation IMiD with a better toxicity profile compared to thalidomide. In the randomized SWOG S0232 phase III trial, RD was shown to be superior to D alone for the treatment of newly diagnosed MM [46]. The CR rate was 22% in the RD arm and 4% in the D arm ($P = 0.001$), with the 1-year PFS rates being 77% and 55%, respectively ($P = 0.002$). The trial was halted after interim analysis due to ethical challenges with using D alone as the control arm and patients were allowed to cross over to RD. Thromboembolic events (TEE) were observed in 20% of patients receiving RD compared to 12% of those receiving D alone. However, thromboprophylaxis with aspirin 325 mg daily was added later in the trial after a high rate of TEE was noted in the RD arm.

Another phase III RCT evaluated whether RlowD (lenalidomide-low-dose dexamethasone) was non-inferior in terms of efficacy and safer compared to RD as an induction

therapy for newly diagnosed MM [47]. Patients were randomized to receive dexamethasone 40 mg for 12 out of 28 days (RD) or 4 out of 28 days (RlowD) along with lenalidomide 25 mg daily from days 1 to 21. After four cycles, the cumulative rate of PR or better was 79% in the RD arm and 68% in the RlowD arm ($P = 0.008$). However, the 1-year respective OS rates in the two arms were 87% and 96% ($P = 0.0002$), mostly driven by the increased mortality during the first 4 months of therapy in the RD arm (5.4%) compared to the RlowD arm (0.45%). Notably, the incidence of grade ≥3 AEs was higher in the RD compared to RlowD arm (52% vs. 35%, respectively; $P < 0.001$), with the most common SAEs being DVT, infection, and fatigue.

In non-TE patients, RD has been shown to be inferior to VRD in terms of PFS and OS in the SWOG S0777 trial [24]. However, survival benefit of novel agent-based triplets over RD has not been shown in TE patients. Patients receiving RD should have stem cells collected after four cycles of therapy due to reports of impaired stem cell mobilization after prolonged exposure to lenalidomide [48, 49].

Stem Cell Transplantation

Stem cell transplantation is an integral part of the treatment backbone in eligible patients with newly diagnosed MM. Several RCTs, both in the context of conventional cytotoxic chemotherapy and novel agents like PIs and IMiDs, have shown ASCT to be an effective consolidative treatment. It improves the depth of response achieved by induction therapy with PIs and IMiDs [25, 50], with a negligible transplant-related mortality (TRM) of around 1% in most studies in the current era.

Transplant Eligibility

There is a lack of clear consensus on strict eligibility criteria for ASCT in MM. However, age and comorbidities are generally taken into consideration while determining transplant eligibility. Age greater than 65 years is considered to be a contraindication for transplant in most countries. However, in the USA, ASCT is frequently offered to patients over 65 years of age in a good functional status and several retrospective studies have shown similar posttransplant PFS and OS in elderly patients who undergo transplantation [51, 52]. There was no significant difference in TRM noted in elderly patients [52]. A prospective French study on MM patients aged 64–74 years has established the feasibility of ASCT in this age group, with 89% of patients undergoing ASCT successfully [53]. Melphalan 200 mg/m^2 was used as a conditioning regimen in 64% and 140 mg/m^2 in 36% of transplanted patients. At 100 days posttransplant, there was no treatment-related mortality observed in this age group. Prior to ASCT, sufficient cardiac, renal, liver, and pulmonary function is also desirable [4]. However, in a CIBMTR study on 1492 patients undergoing ASCT, there was no difference in PFS and OS among patients with and without renal dysfunction. Furthermore, 85% of patients with severe renal insufficiency achieved dialysis independence after ASCT [54]. Hematopoietic cell transplant comorbidity index (HCT-CI) [55] has also been shown to be effective as a risk stratification tool prior to ASCT in MM, with higher HCT-CI scores indicating an inferior survival after transplant [56].

Studies on ASCT in Newly Diagnosed MM (Table 28.4)

The first RCT performing a head-to-head comparison of conventional therapy using alkylating agents with high-dose chemotherapy followed by ASCT (HDT-ASCT) was reported by the IFM group in 1996 [57]. In this study 200 patients were randomized to receive either 18 alternating cycles of VMCP/BVAP (vincristine, melphalan, cyclophosphamide, prednisone/vincristine, carmustine, doxorubicin, prednisone) or 4–6 alternating cycles of VMCP/BVAP immediately followed by HDT-ASCT, using melphalan 140 mg/m^2 and total-body irradiation (TBI; 8Gy) as a conditioning regimen. The ORR was significantly higher in the ASCT arm compared to the conventional therapy arm (81% vs. 57%, respectively; $P < 0.001$), as was the CR rate (22% vs. 5%, respectively; $P < 0.001$). The 5-year PFS rates in the ASCT and conventional therapy arms were 28% and 10% ($P = 0.01$), respectively, and the respective 5-year OS rates were 52% and 12% ($P = 0.03$). TRM was seen in 2% of patients. The MRC Myeloma VII trial also compared alkylating agent-based chemotherapy with ASCT showing similar results, with a significantly superior CR rate, PFS, and OS in the ASCT arm [58]. Death within 100 days of ASCT was seen in 3% of patients in the MRC trial, the cause being sepsis in all but one patient.

With the superiority of HDT-ASCT being established in newly diagnosed MM, the next strategic question was whether all eligible patients must undergo frontline ASCT after induction therapy or can wait for ASCT until their first relapse without compromising survival. To answer this question, Fermand et al. reported a multicenter RCT comparing patients who underwent up-front HDT-ASCT (ASCT arm) with those who had standard-dose therapy (SDT) with the option of salvage ASCT either at first relapse or in the case of primary resistance to SDT (SDT arm) [59]. With a median follow-up of around 6 years, the median PFS in the ASCT arm was 39 months, compared to 13 months in the SDT arm. However, there was no significant difference in OS, with the median OS being 64.6 months in the ASCT arm and

Table 28.4 Trials comparing high-dose therapy with standard-dose therapy in transplant-eligible patients

Author	No.	Treatment schema	Response rate	Long-term outcomes		Ref.
				PFS	OS	
Attal et al.	200	***SDT arm:*** Alternative 3-week cycles of VMCP and BVAP × 12 months (Total of 18 cycles) ***ASCT arm:*** Alternating 3-week cycles of VMCP and BVAP (4–6 cycles) → ASCT (Mel140 + TBI)	≥VGPR rate: SDT: 14% ASCT: 38% ($P < 0.001$)	5-year EFS: SDT: 10% ASCT: 28% ($P = 0.01$)	5-year OS: SDT: 12% ASCT: 52% ($P = 0.03$)	
Child et al.	407	***SDT arm:*** Doxorubicin-carmustine-cyclophosphamide-Melphalan × 4–12 cycles (6-week cycles) → IFN*m* 3× weekly ***ASCT arm:*** Doxorubicin-vincristine-methylprednisolone-cyclophosphamide × 3 cycles or until response attained (3-week cycles) → ASCT (Mel200) → IFN*m* 3× weekly	CR rate: SDT: 8% ASCT: 44% ($P < 0.001$)	Median PFS: SDT: 20 months ASCT: 32 months ($P < 0.001$)	Median OS: SDT: 42 months ASCT: 54 months ($P = 0.04$)	
Fermand et al.	185	***SDT arm:*** SCC by CHOP regimen → VMCP × 6 cycles → option of salvage ASCT for primary resistance or at first relapse ***ASCT arm:*** SCC by CHOP regimen → VAMP × 3–4 cycles → ASCT (lomustine, VP16, cyclophosphamide, Melphalan 140 and TBI)	ORR: SDT: 62% ASCT: 86% CR rate: SDT: 5% ASCT: 19%	Median PFS: SDT: 13 months HDT: 39 months	Median OS: SDT: 64 months HDT: 64.6 months ($P = 0.92$)	
Barlogie et al.	516	***SDT arm:*** VAD × 4 cycles → VBMCP*c* after SCC with high-dose CTX (5-week cycles × 1 year) → IFN*m* or observation × 4 years (option of salvage ASCT at relapse) ***ASCT arm:*** VAD × 4 cycles → SCC with high-dose CTX → ASCT (Mel140 + TBI 12Gy) → IFN*m* or observation	Similar cumulative response rates in both arms	7-year PFS rate: SDT: 16% ASCT: 17% $P = 0.16$	7-year OS rate: SDT: 42% ASCT: 37% $P = 0.78$	
Fermand et al.	190	***SDT arm:*** VMCP (1-month courses) until stable plateau in case of PR or until progression/resistance ***ASCT arm:*** SCC reinforced by cyclophosphamide, doxorubicin, vincristine, and prednisone → VAMP × 3–4 cycles → ASCT (Mel 200 or busulfan + Mel140)	ORR: SDT: 58% ASCT: 83%	Median PFS: SDT: 19 months ASCT: 25 months ($P = 0.07$)	Median OS: SDT: 47.6 months ASCT: 47.8 months ($P = 0.91$)	
Palumbo et al.	273	***SDT arm:*** RD × 4 cycles (28-day cycle) → MPR × 6 cycles (28-day cycle) → R*m* until progression or toxicity or observation ***ASCT arm:*** RD × 4 cycles (28-day cycle) → ASCT × 2 (Mel 200 total) → R*m* until progression or toxicity or observation	CR rate: SDT: 20% ASCT: 16%	Median PFS: SDT: 22 months ASCT: 43 months ($P < 0.001$)	4-year OS rate: SDT: 65% ASCT: 82% ($P = 0.02$)	
Attal et al.	700	***Arm A:*** VRD × 3 cycles → ASCT (Mel200) + VRD × 2 cycles → R*m* × 1 year ***Arm B:*** VRD × 5 cycles → R*m* × 1 year	≥VGPR rate: Arm A: 88% Arm B: 77% ($P = 0.02$)	Median PFS: Arm A: 50 months Arm B: 36 months ($P < 0.001$)	4-year OS rate: Arm A: 81% Arm B: 82% ($P = 0.87$)	

64 months in the SDT arm ($P = 0.92$). Around 90% of patients in the SDT arm who were TE per protocol had successfully received a salvage ASCT. Notably, the average time without symptoms, treatment, or treatment toxicity was higher in the ASCT arm compared to the SDT arm (28 vs. 22 months, respectively).

Subsequently, the phase III US Intergroup trial by Barlogie et al. randomized 516 patients to SDT or HDT-ASCT after induction with VAD and SCC with high-dose cyclophosphamide [60]. Patients in the SDT arm had received consolidation with multiple alkylating agents (VBMCP) for a total of 1 year before being randomized to interferon (IFN)-based maintenance therapy or observation. The conditioning regimen used in the ASCT arm was melphalan 140 mg/m^2 along with total-body irradiation (TBI). The cumulative response rates were identical in both arms. Furthermore, there was no significant difference in the 7-year PFS and OS rates in either arms. Notably, around 55% of patients relapsing in the SDT arm received HDT-ASCT in their first relapse. Treatment-related mortality in the SDT arm was 0.4%, compared to 1.7% in the ASCT arm. A plausible explanation proposed for the lack of superiority of ASCT seen in this study was the use of TBI as a conditioning regimen, which has been shown to be inferior to high-dose melphalan (200 mg/m^2) in MM [4, 61]. Another study comparing frontline ASCT and SDT with alkylating agents showed a nonsignificant trend towards superior PFS in the ASCT arm but no difference in OS in either arms at a median follow-up of 10 years [62]. However, the duration without symptoms, treatment, or

toxicity related to treatment was significantly longer for the ASCT compared to the SDT arm (25 vs. 17 months, respectively; $P = 0.033$).

In the era of PIs and IMiDs, two phase III studies have addressed the question of early versus delayed ASCT thus far. In a study by the Italian group, 273 patients were randomized to receive consolidation with either six cycles of melphalan-prednisone-lenalidomide (MPR) [SDT arm] or tandem autotransplantation with a total melphalan conditioning dose of 200 mg/m^2 (ASCT arm) [63]. All patients had received induction with four cycles of RD prior to randomization. The median PFS in the ASCT and SDT arms were 43 and 22 months, respectively ($P < 0.001$), and the 4-year OS rates in the respective arms were 82% and 65%, respectively ($P = 0.02$). Notably, 63% of patients in the SDT arm received salvage ASCT at relapse. Grade 3/4 hematologic, gastrointestinal, and infectious adverse events were more common in the ASCT arm. A similar question in the context of VRD induction and consolidation therapy has been answered by the phase III IFM study by Attal et al. [50]. In this study, 700 patients were randomized to receive three cycles of VRD induction followed by consolidation with ASCT and two cycles of VRD (Arm A) or five cycles of VRD alone (Arm B). All patients received 1 year of lenalidomide maintenance. A total of 323 out of 350 patients (92%) underwent up-front ASCT in Arm A. Patients undergoing up-front ASCT had a higher VGPR rate compared to those receiving VRD alone (88% vs. 77%, respectively; $P = 0.02$). The rate of MRD negativity by flow cytometry at a sensitivity level of 10^{-4} was also higher in patients receiving early ASCT (79% in Arm A vs. 65% in Arm B; $P = 0.001$). At a median follow-up of 43 months for Arm A and 44 months for Arm B, the median PFS in the respective arms were 50 and 36 months ($P < 0.001$), favoring frontline transplantation. There was no difference in OS at 4 years, which was more than 80% in both arms. Among patients in Arm B who experienced symptomatic relapse necessitating a second-line therapy, 79% were able to successfully undergo a salvage transplant. The rates of grade 3/4 neutropenia, gastrointestinal toxicity, and infections were significantly higher in the group receiving up-front ASCT. There was no significant difference in the overall incidence of second primary malignancies (SPMs) in either arm.

Hence, in the era of PIs and IMiDs, ASCT with high-dose melphalan as a conditioning regimen remains an integral part of treatment and should be offered up front to all eligible patients, based on PFS benefit in both studies [50, 63] and OS benefit in one study [63]. Furthermore, early ASCT has been shown to be cost effective [64] and prolongs the treatment-free interval [59, 62]. Patients with progressive disease after induction therapy also benefit from ASCT [65, 66]. In TE patients who do not undergo frontline ASCT, adequate stem cells should be collected for at least two transplants and ASCT should be offered at first relapse.

Role of Tandem Transplantation

Tandem ASCT is defined as a planned course of treatment with high-dose therapy followed by stem cell infusion within 6 months of the first transplant [4]. The rationale behind a second ASCT is to improve the depth of response and potentially prolong long-term outcomes like PFS and OS. A list of RCTs comparing single and double ASCT in newly diagnosed MM has been summarized in Table 28.5.

In the USA, tandem transplant for newly diagnosed MM was pioneered by the University of Arkansas group. Tandem ASCT was shown to be feasible and superior to historical controls receiving standard therapy in terms of response rate, PFS, and OS [67]. A multicenter randomized trial by the French group (IFM94) in the era of alkylating agent-based induction therapy has shown a superior PFS and OS with double ASCT compared to single ASCT, despite no significant difference in the depth of response [68]. The 7-year OS rates with single and double ASCT in the study were 21% and 42%, respectively ($P = 0.01$), at a median follow-up of over 6 years. Notably, the subgroup of patients who did not achieve at least a VGPR after the first ASCT derived the most benefit from a second ASCT. However, a limitation of this trial was the suboptimal conditioning regimen used in the single-transplant arm. The cumulative melphalan dose in the tandem arm was 280 mg/m^2 compared to 140 mg/m^2 in the single-transplant arm. Seventy-eight percent of patients in the tandem arm were successfully able to undergo two transplantations with no significant difference in hematologic toxicity or TRM compared to patients who received a single transplant. In the HOVON24 trial comparing single and double ASCT after VAD-based induction therapy, the CR rate was significantly higher in patients receiving double transplant [69]. However, there was a marginal difference in the median PFS between the two groups and no significant difference in OS at a median follow-up of 56 months. Similarly, in the Bologna96 randomized trial, there was no significant difference in OS between the single- and double-transplant arms, despite an increase in response rate and PFS duration by 18 months in the double-transplant arm [70]. The cumulative melphalan dose in this trial was 200 mg/m^2 in the single-transplant and 320 mg/m^2 in the double-transplant arm, with no significant difference in TRM in the two arms. Similar to the IFM94 trial [68], patients who did not achieve nCR after the first ASCT benefitted the most from a second transplant, with a trend towards OS benefit in this subgroup of patients.

Two randomized trials have compared single and double transplants in the context of PI and IMiD-based induction and maintenance therapy. The StaMINA trial randomized 758 TE patients who had received 2–12 months of induction therapy to receive either a single ASCT (Arm A), single ASCT followed by four cycles of VRD consolidation therapy (Arm B), or tandem ASCT (Arm C) [71]. The conditioning

Table 28.5 Studies comparing single- with double-autologous stem cell transplantation

Author	No.	Treatment schema	Response rate	Long-term outcomes		Ref.
				PFS	OS	
Attal et al.	399	***Induction:*** VAD × 3–4 cycles (3-week cycles) ***Transplant:*** S-ASCT (Mel140 + TBI 8Gy) or D-ASCT (Mel140 for 1st and Mel140 + TBI 8Gy for 2nd) ***Maintenance:*** IFN 3× weekly initiated after hematologic reconstitution	≥VGPR rate: S-ASCT: 42% D-ASCT: 50% ($P = 0.01$)	7-year PFS rate: S-ASCT: 10% D-ASCT: 20% ($P = 0.03$)	7-year OS rate: S-ASCT: 21% D-ASCT: 42% ($P = 0.01$)	
Sonneveld et al.	303	***Induction:*** VAD × 3–4 cycles (3-week cycles) ***Transplant:*** S-ASCT (Mel70 × 2) or D-ASCT (Mel70 × 2 + CTX 120 mg/kg + TBI) ***Maintenance:*** IFN*m* in both arms	CR rate: S-ASCT: 13% D-ASCT: 28% ($P = 0.002$)	Median PFS: S-ASCT: 23 months D-ASCT: 24 months ($P = 0.032$)	Median OS: S-ASCT: 55 months D-ASCT: 50 months ($P = 0.39$)	
Cavo et al.	321	***Induction:*** VAD × 4 cycles (28 day cycles) ***Transplant:*** S-ASCT (Mel200) or D-ASCT (Mel200 for first and Mel120 + busulfan 12 mg/kg for second) ***Maintenance:*** IFN*m* in both arms	≥nCR rate: S-ASCT: 33% D-ASCT: 47% ($P = 0.008$)	Median PFS: S-ASCT: 24 months D-ASCT: 42 months ($P < 0.001$)	7-year OS rate: S-ASCT: 46% D-ASCT: 43% ($P = 0.90$)	
Stadmauer et al.	758	***Induction:*** 2–12 months of induction therapy (regimen not specified) ***Arm A:*** ASCT (Mel200) → R*m* until progression ***Arm B:*** ASCT (Mel200) → VRD × 4 cycles → R*m* until progression ***Arm C:*** ASCT (Mel200 × 2) → R*m* until progression	NR	38-month PFS rate: Arm A: 52% Arm B: 57% Arm C: 56% (P = NS)	38-month OS rate: Arm A: 83% Arm B: 86% Arm C: 82% (P = NS)	
Cavo et al.	614	***Induction:*** VCD → 3–4 cycles ***Transplant:*** Single or double ASCT ***Maintenance:*** R*m* until progression or toxicity	NR	3-year PFS rate: S-ASCT: 60% D-ASCT: 73% ($P = 0.03$)	NR	

dose of melphalan was 200 mg/m^2 for each transplant. All arms received lenalidomide maintenance until progression. At a median follow-up of 38 months, there was no significant difference between the PFS and OS rates in the three arms. The 38-month probabilities of OS was 83%, 86%, and 82% in Arm A, Arm B, and Arm C, respectively. The cumulative incidences of first SPM were 4%, 6%, and 5.9%, respectively, in the three arms. The phase III EMN02/HO95 trial randomized 614 TE patients to either VMP (bortezomib-melphalan-prednisone) consolidation, single-ASCT, or double-ASCT arm after induction therapy with 3–4 cycles of VCD [72]. All patients received lenalidomide maintenance until progression or toxicity. At a median follow-up of 27 months, the 3-year PFS rates in patients receiving a single and double ASCT was 60% and 73%, respectively ($P = 0.03$). The PFS benefit of double ASCT was evident in all predefined subgroups, including those with high β-2 microglobulin, bone marrow plasma cells >60%, lactate dehydrogenase above upper normal limit, revised ISS-II, and high-risk cytogenetic abnormalities by FISH. OS data was not mature at the time the study was reported. Based on these studies, there is a lack of convincing evidence to perform tandem ASCT in the era of PI- and IMiD-based induction and post-transplantation therapies. Randomized trials are currently under way to determine whether patients achieving less than a VGPR after the first transplant can benefit from a second transplant in the novel agent era.

Optimal Conditioning Regimen

The IFM group had tested high-dose melphalan (200 mg/m^2) with low-dose melphalan (140 mg/m^2) plus 8 Gy total-body irradiation (TBI) as a conditioning regimen in 282 patients in the context of induction therapy with four cycles of VAD [61]. Patients receiving high-dose melphalan had a superior 45-month OS rate of 66%, compared to 46% in patients receiving low-dose melphalan plus TBI ($P = 0.05$). Furthermore, patients receiving high-dose melphalan had a faster hematologic recovery, shorter duration of hospitalization, and lower rates of severe oral mucositis. This study paved the way for high-dose melphalan to be the optimal conditioning regimen in MM.

Recently, there has been some evidence on the benefit of combining bortezomib with melphalan during the conditioning phase [73, 74]. Administration of bortezomib within 24 h

following melphalan was shown to induce robust plasma cell apoptosis in pharmacodynamics studies, with 51% achieving VGPR or better [73]. A phase II IFM study on the combination of melphalan 200 mg/m^2 with bortezomib at a cumulative dose of 4 mg/m^2 (Mel-Vel) showed a post-ASCT VGPR rate of more than 70%, with no increase in hematologic toxicity [74]. A matched control analysis with patients receiving melphalan alone showed that Mel-Vel led to higher rates of CR after transplant. However, RCTs comparing melphalan alone or combined with novel agents are needed before incorporation of such strategies in practice. Other alkylating agents like busulfan in addition to melphalan have shown PFS benefit but no difference in OS, with a higher TRM [75, 76]. Hence, high-dose melphalan (200 mg/m^2) remains the standard of care currently, except in patients with renal dysfunction where lower dose (140 mg/m^2) should be used [19].

Stem Cell Mobilization

The three most common stem cell mobilization strategies include growth factor, growth factor plus plerixafor, and chemotherapy. Strategies for mobilization vary among different institutions. Growth factor (granulocyte-colony-stimulating factor [G-CSF]) is one of the most common mobilization strategies currently. Factors predicting suboptimal mobilization with G-CSF include advanced age, low platelet count at mobilization, use of filgrastim instead of pegfilgrastim, and longer duration of lenalidomide therapy prior to mobilization [77]. Plerixafor, when added to G-CSF, leads to adequate stem cell mobilization in patients receiving lenalidomide-based induction therapy [78]. However, the efficacy of added plerixafor is similar to adding low-dose cyclophosphamide (1.5 mg/m^2) to G-CSF in the novel agent era [79]. Hence, due to low cost of cyclophosphamide, it should be preferred over plerixafor for use in conjunction with G-CSF in patients with prior exposure to lenalidomide. The threshold of stem cell infusion dose for a single transplant in Mayo Clinic is 3×10^6 CD34+ cells/kg [80].

Role of Allogeneic Transplantation

With the advent of effective and well-tolerated anti-myeloma agents and excellent activity of ASCT as a consolidative therapy, allogeneic stem cell transplantation (Allo-SCT) is not routinely used in newly diagnosed MM. In the IFM trials comparing ASCT → Allo-SCT (auto-allo) with tandem ASCT after induction therapy with VAD, the median PFS was similar in both arms and there was a nonsignificant towards a superior OS in the tandem ASCT arm [81]. Notably, 24% of patients developed grade 2–4 acute graft-versus-host disease (GVHD) after allo-SCT and 36% of evaluable patients developed extensive chronic GVHD. Another study on 162 TE newly diagnosed MM patients biologically randomized to either a non-myeloablative allo-SCT or ASCT after induction therapy with VAD and a single ASCT showed a superior PFS and OS in the allo-SCT arm [82]. The BMT-CTN and PETHEMA trials have shown comparable survival between ASCT and Allo-SCT, with a higher rate of TRM in the allo-SCT arms [83, 84]. Due to a high treatment-related morbidity and mortality, allo-SCT in newly diagnosed MM should be restricted to clinical trials in high-risk patients and can be an option in certain situations, including primary refractory disease or lack of response to ASCT [4].

Post-transplantation Maintenance and Consolidation

Despite increasing the depth of response and the rate of MRD negativity, ASCT is not curative in MM and most patients eventually relapse. Hence, post-transplantation maintenance or consolidation therapy is administered with the intent of further improving the depth and prolonging the duration of response, to eventually improve long-term outcomes, including PFS and OS. A pooled analysis of studies involving head-to-head comparison of continuous and fixed-duration therapy in MM has shown a superior PFS and OS with continuous therapy, with the 4-year OS rate being 69% with continuous and 60% with fixed-duration therapy ($P = 0.003$) [85]. Maintenance therapy involves prolonged treatment with an anti-myeloma agent, usually until progression or unacceptable toxicity. Consolidation involves a short course of single agent or combination therapy for a fixed duration after ASCT [86].

Prior to the introduction of PIs and IMiDs, glucocorticoid [87] and interferon [88, 89] were used for posttransplant maintenance therapy. Two large meta-analysis of interferon maintenance showed an OS benefit ranging from 4 to 7 months [88, 89]. However, currently, there is no role of interferon-based maintenance therapy in MM due to the toxicity profile and its impact on quality of life. With the advent of novel agents, thalidomide was tested as a maintenance therapy in various randomized trials [90–97]. A meta-analysis however showed that thalidomide maintenance in the context of ASCT significantly improved PFS (HR 0.67, 95% CI, 0.61–0.74; $P < 0.001$), but not OS (HR 0.90, 95% CI, 0.73–1.11; $P = 0.343$) [98]. The toxicity profile of thalidomide is unfavorable for long-term maintenance therapy due to high rates of PN and poor health-related quality of life (HRQOL) [97]. Furthermore, the UK MRC IX trial has shown worse OS with thalidomide maintenance in patients with high-risk cytogenetic abnormalities by FISH ($P = 0.01$)

Table 28.6 Phase III studies on lenalidomide and bortezomib maintenance

Author	No.	Maintenance dose/regimen	Long-term outcomes		Ref.
			PFS	OS	
McCarthy et al.	460	Lenalidomide 10 mg daily (range, 5–15 mg) until progression	Median PFS (month): Lenalidomide: 46 Placebo: 27 ($P < 0.001$)	3-year OS rate (%): Lenalidomide: 88 Placebo: 80 ($P = 0.03$)	
Attal et al.	614	Lenalidomide 10 mg daily × 3 months → 15 mg thereafter if tolerated until progression	Median PFS (month): Lenalidomide: 41 Placebo: 23 ($P < 0.001$)	3-year OS rate (%): Lenalidomide: 80 Placebo: 84 ($P = 0.29$)	
Palumbo et al.	251	Lenalidomide 10 mg daily days 1–21 (28-day cycle) until progression	Median PFS (month): Lenalidomide: 42 Placebo: 22 ($P < 0.001$)	3-year OS rate (%): Lenalidomide: 88 Placebo: 79 ($P = 0.14$)	
Jackson et al.	828	Lenalidomide until progression	Median PFS (month): Lenalidomide: 50 Placebo: 28 ($P < 0.001$)	NR	
Sonneveld et al.	827	Bortezomib 1.3 mg/m^2 every other week or thalidomide 50 mg daily for 2 years	Median PFS (month): Bortezomib: 35 Thalidomide: 28 ($P = 0.002$)	5-year OS rate: Bortezomib: 61% Thalidomide: 55% ($P = 0.07$)	

[99]. Hence, lenalidomide has largely replaced thalidomide as a posttransplant maintenance therapy due to better tolerability and data on improved OS, which will be described below.

Here, we discuss the lenalidomide and bortezomib-based maintenance strategies in MM in the posttransplant setting. The phase III studies on lenalidomide and bortezomib maintenance have been summarized in Table 28.6.

Lenalidomide Maintenance

Lenalidomide has been shown to be an effective maintenance therapy in MM in the posttransplant setting in four independent phase III RCTs [63, 100, 101]. The CALGB100104 was the first US trial showing a survival advantage with lenalidomide maintenance [100]. In this trial, 460 patients who had a stable disease or better 100 days post-transplantation were randomized to receive lenalidomide or placebo at a starting dose of 10 mg/day (range, 5–15 mg). Induction regimen other than bortezomib, lenalidomide, or thalidomide prior to ASCT was used in only 6% of patients. Lenalidomide was administered until disease progression or unacceptable toxicity. At a median follow-up of 34 months, the median PFS of patients in the lenalidomide group was 46 months compared to 27 months for those in the placebo group ($P < 0.001$). Furthermore, the 3-year OS rate was also significantly superior in the lenalidomide compared to the placebo group (88% vs. 80%, respectively: $P = 0.03$). Grade 3/4 hematologic AEs were significantly higher in the lenalidomide compared to the placebo group as expected. The cumulative incidence of SPMs was 8% in the lenalidomide and 3% in the placebo group ($P = 0.008$). The simultaneously released IFM trial randomized 614 patients with nonprogressive disease after ASCT to lenalidomide maintenance or placebo [101]. The starting dose of lenalidomide was 10 mg daily for the first 3 months, with subsequent escalation to 15 mg if well tolerated. Approximately 50% of patients had received VAD induction therapy pre-ASCT and the other half received VD. Maintenance was continued until relapse. At a median follow-up of 30 months, the median PFS was higher in the lenalidomide compared to the placebo group (41 vs. 23 months, respectively; $P < 0.001$). However, there was no significant difference in the 3-year OS rates, which was more than 80% in both arms. The cumulative incidence of SPMs was 3.1 per 100 patient-years in the lenalidomide and 1.2 per 100 patient-years in the placebo group ($P = 0.002$). Grade 3/4 hematologic toxicity including thromboembolic events was more frequent in the lenalidomide group, with no difference observed in the rate of grade 3/4 PN. The GIMEMA trial randomized 251 patients to lenalidomide or no maintenance after induction therapy with four cycles of RD and consolidation with either six cycles of MPR or tandem ASCT [63]. At a median follow-up of 51 months, the median PFS was significantly longer with lenalidomide maintenance compared to no maintenance (42 vs. 22 months, respectively; $P < 0.001$). However, there was no significant difference in the 3-year OS rates in the two arms. Grade 3/4 hematologic and dermatologic toxic effects were significantly higher in the lenalidomide arm.

A prospectively planned individual patient-level meta-analysis of the three studies (CALGB, IFM, and GIMEMA) showed a significant OS benefit with lenalidomide maintenance compared to no-maintenance therapy [5]. The 7-year OS rate was 62% with lenalidomide maintenance versus 50% with no-maintenance therapy ($P = 0.001$) at an updated median follow-up of 80 months. The OS benefit was evident in most subgroups; however, patients with ISS stage III disease and high-risk cytogenetics did not derive survival benefit from lenalidomide maintenance. The cumulative incidence of both hematologic and solid SPMs was higher with lenalidomide maintenance therapy. Notably, the HRQoL score of patients receiving lenalidomide maintenance in the real world has been shown to be similar to those receiving no-maintenance therapy [102].

The Myeloma XI study from the UK had randomized patients to lenalidomide maintenance or observation after induction therapy with CRD (cyclophosphamide-lenalidomide-dexamethasone) or CTD (cyclophosphamide-thalidomide-dexamethasone) and consolidation with ASCT. At a median follow-up of 36 months, the median PFS was 50 months in patients receiving lenalidomide maintenance compared to 28 months in those on observation. Furthermore, the PFS benefit with lenalidomide maintenance was evident in all prespecified subgroups, including in those with high-risk cytogenetic abnormalities and ISS stage III disease. The most common grade 3/4 AE was neutropenia.

In summary, given the OS benefit noted in the meta-analysis including three large RCTs, lenalidomide maintenance is currently the standard of care in all patients posttransplant. However, OS benefit has not yet been demonstrated in patients with high-risk cytogenetic abnormalities, in whom bortezomib maintenance should be preferred based on data from the HOVON trial, which will be described below. Furthermore, it is unclear whether a fixed duration of lenalidomide maintenance after ASCT is as good as prolonged maintenance until disease progression, and it needs to be answered by future RCTs.

Bortezomib Maintenance

There is a lack of placebo-controlled randomized trials evaluating bortezomib maintenance after ASCT in literature. However, the HOVON-65/GMMG-HD4 trial performed a head-to-head comparison of bortezomib-based induction and maintenance therapy with VAD induction followed by posttransplant maintenance with thalidomide [33]. It should be noted that patients were not randomized to bortezomib or thalidomide maintenance after ASCT and the study was not designed for direct comparison between bortezomib and thalidomide. Nevertheless, patients receiving bortezomib-based induction and maintenance therapy had a superior OS in the entire cohort and also in the subgroups with deletion (17p) and renal failure. Notably, in patients with deletion (17p), the median PFS was 18 months without bortezomib and 36 months with bortezomib-based therapy, which also translated into a superior OS [3-year OS 83% with bortezomib and 36% without bortezomib in deletion (17p) subgroup; $P < 0.001$] [34]. An updated report of this trial at a median follow-up of more than 7 years showed that the negative prognostic impact of deletion (17p) was abrogated in the bortezomib arm but not in the standard arm [103]. The plausible explanation for the excellent activity of bortezomib in deletion (17p) patients is the induction of apoptosis in myeloma cells by altering the balance between proteasome load and proteasome capacity in a p53-independent fashion [104]. The median duration of bortezomib maintenance at a starting dose of 1.3 mg/m^2 every other week was 23 months, compared to 14 months for thalidomide, indicating that bortezomib was well tolerated and feasible for long-term posttransplant maintenance therapy. Emergence of grade 3/4 PN during bortezomib maintenance therapy was seen in 5% of patients, with the rate of treatment discontinuation due to any toxicity being 11%.

Hence, lenalidomide should be used for posttransplant maintenance in the vast majority of MM patients, except in those with high-risk cytogenetics especially deletion (17p) and t(4;14), where bortezomib maintenance should be considered.

Posttransplant Consolidation Therapy

The goal of consolidation therapy in MM is to improve the depth of response and possibly long-term outcomes. A phase III trial evaluating VTD versus TD consolidation after ASCT showed a significant increase in CR rates after consolidation with VTD, which translated into a superior PFS but not OS at a median follow-up of 30 months [105]. Grade 2/3 PN was more frequent with VTD compared to TD (8.1 vs. 2.4%, respectively). Notably, high-risk cytogenetic abnormalities, including deletion(17p) and/or t(4;14), retained their negative prognostic impact on PFS in the TD arm but not in the VTD arm. Another study by the Nordic myeloma group randomized 370 patients 3 months after ASCT to receive either 21 weeks of bortezomib or no-consolidation therapy [106]. Consolidation with bortezomib led to an improvement in PFS by 7 months ($P = 0.05$), but did not have any impact on OS. Pooled results from two phase III studies comparing bortezomib consolidation and observation after ASCT in MM also showed only PFS but no OS benefit, with a significant PFS benefit seen only in patients achieving less than a VGPR after ASCT [107]. Finally, the StaMINA trial on 758 TE patients in the era of novel agent-based induction and post-transplantation therapy did not show any PFS or OS benefit from four cycles of VRD consolidation after ASCT [71].

Based on the data presented above, there is a lack of convincing evidence for use of consolidation therapy after ASCT, especially in the context of PI- and/or IMiD-based induction therapy and lenalidomide maintenance until progression.

Supportive Care and Toxicities

Peripheral Neuropathy

Peripheral neuropathy is one of the most common serious AEs of PI bortezomib and is also seen with IMiDs thalidomide and lenalidomide. Drug-induced PN by bortezomib or thalidomide can present with sensory, motor, and autonomic symptoms, with the potential targets being small fibers, dorsal root ganglia, and afferent sensory and efferent motor fibers [108]. Important predisposing conditions for drug-induced neuropathy are preexisting PN and medical comorbidities, including diabetes mellitus, vitamin deficiencies, alcohol abuse, or viral infections [108]. Subcutaneous bortezomib has been shown to be non-inferior to intravenous bortezomib in terms of efficacy, with a lower incidence of grade 2–4 PN (24% with subcutaneous and 41% with intravenous formulation; $P = 0.012$) [109]. Changing the route of administration of bortezomib to subcutaneous and the frequency from twice weekly to once weekly have led to deceased rates of bortezomib-induced PN (BiPN) in clinical practice. For moderate BiPN (grade 2), dose should be reduced from 1.3 to 1 mg/m^2, and for severe PN (grade 3), dose should be temporarily withheld and can be resumed at a lower dose of 0.7 mg/m^2, once symptoms resolve. Bortezomib should be discontinued if patients develop a disabling PN (grade 4) [110]. BiPN has been shown to resolve or improve in about two-thirds to three-quarters of patients, with median time for reversal or improvement being 2–3 months. On the other hand, recovery in thalidomide-induced PN (TiPN) is limited to about a quarter of patients and takes around 4–6 years [108]. Hence, dose modification guidelines should be strictly followed in the setting of drug-induced PN to maximize the chances of reversal or improvement.

Infections

The 2017 NCCN guidelines state that prophylaxis for *Pneumocystis jirovecii* pneumonia (PJP) can be considered in myeloma patients [4]. Similarly, intravenous immunoglobulin should be considered in life-threatening infections [4]; however, routine use is not recommended [17]. In the phase III APEX study comparing bortezomib-dexamethasone with dexamethasone alone, the incidence of herpes zoster virus infections was significantly higher in the bortezomib arm [111], which led to the recommendation of using acyclovir or valacyclovir prophylaxis in all patients receiving PI-based therapies [17]. Immediate therapy with broad-spectrum antibiotics and supportive care is needed.

Thromboembolic Phenomenon

Patients with MM have a higher base rate of thromboembolic phenomena compared to general population, with the baseline risk of venous thromboembolic (VTE) events being 3–4% [17]. The rate of thrombosis has been shown to be higher in patients receiving high-dose dexamethasone and IMiDs. The ECOG trial comparing RD with RlowD in newly diagnosed MM showed greater than a twofold higher rate of thromboembolic phenomena in the high-dose dexamethasone compared to the low-dose dexamethasone arm (26% vs. 12%, respectively; $P = 0.0003$) [47]. This study led to the routine use of low-dose dexamethasone in combination regimens. Aspirin at a dose of 100 mg daily is currently recommended for VTE prophylaxis in myeloma patients on IMiDs [4, 17, 112]. Full-dose anticoagulation with warfarin or low-molecular-weight heparin should be initiated in the setting of a clot, without any urgent need to discontinue IMiDs.

Bone Disease

Osteolytic bone disease due to the activation of osteoclasts is one of the most common clinical manifestations of MM and also causes significant worsening of HRQoL [113]. Bisphosphonates are the mainstay of therapy for myeloma bone disease, with their mechanism of action being inhibition of osteoclasts and subsequently bone resorption. A double-blind placebo-controlled trial on 392 MM patients with at least one lytic bone lesion showed a significantly reduced incidence of skeletal events in patients receiving pamidronate compared to those receiving placebo (24% vs. 41%; $P < 0.001$) [114]. Furthermore, pamidronate was well tolerated and led to decrease in bone pain. Another double-blind RCT on patients with hypercalcemia of malignancy compared zoledronic acid and pamidronate head-to-head [115]. Zoledronic acid was superior to pamidronate in terms of the rate of normalization of serum calcium level by day 4 and duration of response. The UK MRC Myeloma IX trial comparing zoledronic acid with clodronic acid in both TE and non-TE newly diagnosed MM patients showed a significant improvement in the median OS by 5.5 months in the zoledronic acid arm [116]. Current NCCN guidelines

recommend addition of bisphosphonates to the treatment regimen in all symptomatic MM patients, regardless of the evidence of bony lesions. Monitoring of renal function, baseline dental examination, and monitoring for osteonecrosis of jaw while on bisphosphonates are also recommended [4].

Miscellaneous

In patients with myeloma-associated anemia, erythropoietin therapy can be used [117, 118] to maintain a target hemoglobin around 12 gm/dl. Granulocyte colony-stimulating factor (G-CSF) may be administered in patients with severe neutropenia during therapy [17]. In patients with symptomatic hyperviscosity, plasmapheresis can be used to reduce the risk of end-organ damage [4, 119]. For impending spinal cord compression, high-dose dexamethasone and radiation therapy should be emergently initiated [17]. Orthopedic consultation should be sought for pathological fractures. Certain patients with symptomatic vertebral compression fractures might benefit from kyphoplasty or vertebroplasty [4].

Bortezomib-based therapy should be used initially in those with renal insufficiency since bortezomib does not need dose modification in renal dysfunction [19]. Furthermore, it leads to a rapid reduction of tumor burden, which decreases the nephrotoxic effects of paraproteins. In TE patients with renal dysfunction, melphalan should be used at a dose of 140 mg/m^2 [19]. A high-cutoff hemodialysis (HD) technique to remove free light chains has shown a higher rate of dialysis independence compared to HD with conventional high-flux dialyzers in MM patients presenting with myeloma cast nephropathy receiving initial therapy with bortezomib-dexamethasone [120].

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Role of Hematopoietic Cell Transplantation for Myeloma

29

Heather Landau and Sergio Giralt

Introduction and Historical Perspective

More than 30 years ago McElwain and Powles demonstrated that dose intensification of melphalan could result in significant responses in patients with relapsed and refractory multiple myeloma (MM) [1]. However, significant myelosuppression occurred which limited the utility of this treatment strategy [2]. Barlogie et al. demonstrated that high-dose melphalan therapy followed by infusion of autologous bone marrow was feasible and resulted in predictable hematologic recovery within 28 days [3]. Subsequent confirmatory trials in the setting of relapsed disease were followed by the use of high-dose therapy (HDT) with autologous hematopoietic cell transplantation (HCT) as consolidation of initial remissions in patients with newly diagnosed MM [4–14]. Randomized trials in the front-line setting compared treatment outcomes of patients receiving multiagent chemotherapy regimens (usually combinations of vinca alkaloids, anthracyclines, alkylators, nitrosoureas, and steroids) to patients who received consolidation with HDT and auto-HCT [7–14]. The preponderance of evidence showed that HDT was associated with improved outcomes including event-free (EFS), progression-free (PFS), and in some but not all studies overall survival (OS) [7–14]. Depth of response, particularly achievement of a complete response (CR), was associated with longer PFS and OS in MM and was likely responsible for the initial success of HDT and autologous HCT [15].

The advent of proteasome inhibitors (bortezomib and carfilzomib) and the immunomodulatory (IMID) drugs (thalidomide and lenalidomide) have rendered all prior randomized trials of HDT versus chemotherapy in MM less relevant since induction therapy with these agents has been shown to be associated with more frequent and deeper responses than the traditional vincristine, adriamycin, and dexamethasone (VAD) induction [16]. In this chapter we review the basics of HCT for myeloma, current results, and future direction of this important treatment modality.

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Basics of Hematopoietic Cell Transplantation (HCT) for Myeloma [17]

HCT is a complex procedure. The patient receives a combination of chemical and physical agents to eliminate a malignant disorder or a poorly functioning bone marrow supported by reinfusion of HSC from the patient or a third-party source (related or unrelated). As with solid organ transplantation HCT candidates should meet a set of organ function and psychosocial criteria that may vary from transplant center to transplant center but are aimed at determining the risk-benefit ratio of HCT versus other treatment approaches.

Depending on the source of stem cells, HCT can be categorized as either autologous (hematopoietic stem cells (HSCs) are obtained from patient) or allogeneic (HSCs are obtained from a third party). HSCs can be obtained from the marrow cavity or can be mobilized in large quantities into the peripheral blood using medications such as filgrastim or plerixafor and collected through apheresis techniques.

Transplant Eligibility

MM is the most common indication for autologous HCT in the world today with more than 6000 procedures performed in the United States every year [18]. Which MM patient is an appropriate candidate for HCT has been a subject of continued discussion in the HCT and MM literature. Table 29.1 lists the current criteria being utilized to determine HCT eligibility for both autologous and allogeneic HCT in MM patients according to recently published guidelines from the American Society of Blood and Marrow Transplantation (ASBMT) [19].

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Table 29.1 Criteria used to determine HCT eligibility for MM

Criteria	Autologous HCT	Allogeneic HCT	Comments and references
Age	No age limit but rarely performed over age 80	No age limit but rarely performed over age 70	[18–20]
Performance status	KPS ≥ 70	KPS ≥ 80	[18, 21]
HCT-CI	No limit	≤ 1	Patients with KPS < 80 and high HCT-CI should be considered for alternative therapies rather than autologous HCT
Response to therapy	At least a PR	At least a PR	Patients with "primary" refractory disease are an exception for autologous HCT
Social support	Required	Required	Older myeloma patients undergoing HCT may require help for activities of daily living during the early post-HCT period to ensure compliance with medications and adequate hydration and nutrition
Patient wishes	Required	Required	

Initially limited to younger patients (less than 65 years of age), multiple retrospective analyses have demonstrated that outcomes for patients over the age of 65 are not statistically significantly different than outcomes for younger patients and thus age alone should not be considered a contraindication for HCT [20–24]. Notwithstanding, few autografts are performed in patients over the age of 80 and few allografts for MM are performed in patients over 70 years of age.

The hematopoietic cell transplantation comorbidity index (HCT-CI) developed by Sorror et al. to predict nonrelapse mortality after allogeneic HCT incorporates various measurements of organ function (cardiac, pulmonary, renal, hepatic) together with existence of other comorbidities into one score. Patients with HCT-CI scores of 0 to 1 have significantly less NRM than those with scores of 2 or greater with either a myeloablative or reduced-intensity conditioning regimen (RIC) [25].

The HCT-CI has been validated in the setting of autologous HCT and is a valuable tool to determine the risk–benefit ratio of this procedure. The Center for International Blood and Marrow Transplant Research (CIBMTR) recently analyzed the outcomes of 1156 MM patients reported to the registry who underwent auto-HCT after high-dose melphalan. NRM rates were similar for patients with HCT-CI scores of 0 to 1 versus 2 or greater and were 2%. However, on multivariate analysis, OS was inferior in groups with HCT-CI score of 1 to 2 (relative risk, 1.37, [95% CI, 1.01 to 1.87]; $P = 0.04$) and HCT-CI score of greater than 2 (relative risk,1.5 [95% CI, 1.09–2.08]; $P = 0.01$). OS was also inferior with Karnofsky performance status <90 ($P < 0.001$) [26]. Patients with KPS of less than 80 and high HCT-CI had 100-day NRM rates after HDT with autologous HCT of approximately 10%. Therefore the risk–benefit ratio of HDT should be balanced against the benefits of continued nontransplant therapies. In general, for older patients with high HCT-CI and KPS of less than 80 we have recommended proceeding to autologous HCT only if they have failed to achieve at least a very good partial response (VGPR) to induction therapy. End-stage renal disease is not an absolute contraindication to HCT in MM with many centers routinely performing autografting for MM in patient dialysis [27, 28].

Although response to induction therapy has been associated with HCT outcomes most retrospective data suggests that patients with primary induction failure (i.e., less than a partial response to induction therapy) still benefit from HDT with autologous HCT [29–32]. Whether this still holds true for patients failing modern induction therapy is uncertain but retrospective analysis of CIBMTR data suggests that although additional lines of therapy may improve response prior to autologous HCT this did not result in improved outcomes over proceeding directly to HDT [33].

Stem Cell Procurement

Autologous HSCs for MM patients are most frequently obtained through peripheral blood stem cell mobilization utilizing either chemotherapy in combinations with cytokines or cytokines alone. In North America autologous HSC mobilization is most commonly performed with granulocyte colony-stimulating factor (GCSF-filgrastim) alone or in combination with the CXCR4 antagonist plerixafor [34, 35]. In a randomized trial the combination of plerixafor and GCSF resulted in significant improvement in stem cell collection yields a total of 106 of 148 (71.6%) patients in the plerixafor group and 53 of 154 (34.4%) patients in the placebo group met the primary endpoint ($P < 0.001$). A total of 54% of plerixafor-treated patients reached target after one apheresis, whereas 56% of the placebo-treated patients required four aphereses to reach target [36]. Because of cost considerations many centers only use plerixafor in the event that CD34 peripheral blood counts are low (usually less than 10 CD34 cells/microliter) after 4 days of filgrastim therapy. This "just-in-time" plerixafor strategy has been shown to be cost effective [37]. A number of risk factors for poor mobilization in MM patients have been described and are summarized in Table 29.2.

Conditioning Regimen

The conditioning regimen is the combination of agents given to eliminate malignant cells exploiting the dose–response phenomena that most cancer cells exhibit and in the setting of allogeneic SCT suppress the host immune system to allow engraftment of donor cells. For allogeneic HCT conditioning regimen intensity has been classified according to their myelosuppressive effects into myeloablative, reduced intensity, and non-myeloablative [17].

For MM the most commonly utilized conditioning regimen for autologous HCT is melphalan 200 mg/m^2 throughout the world. Moreau et al. performed the only randomized trial comparing melphalan 200 mg/m^2 to melphalan and total-body irradiation. A total of 282 evaluable patients were randomized. The median duration of event-free survival was similar in both arms (21 vs. 20.5 months, $P = 0.6$), but the 45-month survival was 65.8% for high-dose melphalan and 45.5% for melphalan and total-body irradiation (TBI) ($P = 0.05$) [38]. The addition of busulfan or bortezomib to the standard melphalan has in retrospective analysis been proposed as potentially superior than high-dose melphalan but prospective comparative trials need to be done [39–42].

For allogeneic HCT, RIC is the most frequently utilized unless in the context of a CD34 selected peripheral blood stem cell graft (PBSC) [43]. However, the role of myeloablative conditioning for allografting continues to be explored particularly in the context of CD34 selected allografts [44].

Table 29.2 Risk factors for poor mobilization in myeloma patient

Patient factors	Treatment factors	Procedure factors
Age—older age reduces mobilization yields	Treatment with melphalan or bendamustine	Poor catheter flow
Extent of marrow infiltration	More than four cycles of lenalidomide	Low apheresis volumes
Platelet count (measurement of marrow reserve)	Radiation to pelvis- or marrow-bearing bones	
	Extensive prior therapy	

Complications of High-Dose Therapy in Myeloma

HDT is associated with significant morbidity due to the effects of intense myelosuppression and the effects of the conditioning regimen on normal tissues. The HCT procedure can be divided into five phases [17]:

Phase I: Chemotherapy phase
Phase II: Cytopenic phase
Phase III: Early recovery phase
Phase IV: Early convalescence phase
Phase V: Late convalescence

Although considered the least intense and less toxic of all conditioning regimens high-dose melphalan is associated with a variety of complications that are summarized in Table 29.3.

Despite being associated with low mortality rates (less than 3%), high-dose melphalan is associated with significant morbidity and a high treatment-related symptom burden as documented by Campagnaro et al. Fatigue, insomnia, loss of appetite, weakness, and feeling sick were the most common described symptoms that interfered with patients' quality of life and activity level. The peak symptom burden

Table 29.3 Randomized trials of early vs. delayed HCT in up-front therapy for myeloma

Author (ref)	*N*	Median PFS	OS at 4 years	Comments
Palumbo (2014)	273	HDT 43.3 months No HDT 22.4 months	HDT 81.6% No HDT 65.3%	No proteosome inhibitor Second randomization to lenalidomide maintenance
Gay (2015)	389	HDT 43.3 months No HDT 28.6 months	HDT 77% No HDT 68%	No proteosome inhibitor exposure
Attal (2015)	700	HDT Not reached 61% at 3 years Delayed HDT 46% at 3 years	3-year OS 88% for both groups	
Sonneveld (2016)	1510	HDT Not reached No HDT 44 months	HDT Not reached No HDT Not reached No difference	Induction CyBorD Consolidation VMP All groups benefitted from HCT

occurred at the time of white blood cell count nadir and approximately a third of the patients had not recovered to baseline a month posttransplant [45]. Increased plasma levels of interleukin 6 have been correlated with increasing symptom burden and could be a potential target for reduction of symptom burden [46].

Current Controversies in HCT for Myeloma

Optimal Induction Regimen

The optimal induction regimen should be effective, well tolerated, spare hematopoietic stem cell, and not negatively impact HDT outcomes. A detailed discussion of optimal induction therapy for MM patients is addressed in other chapters of this book. In brief, randomized trials have demonstrated that for transplant-eligible patients optimal induction therapy will include a IMID (thalidomide or lenalidomide) and a proteosome inhibitor (bortezomib or carfilzomib). Deeper and quicker responses are typically achieved with three-drug regimens such as thalidomide-bortezomib-dexamethasone (VTD), cyclophosphamide-bortezomib-dexamethasone (CyBorD), bortezomib-adriamycin-dexamethasone (PAD), or lenalidomide-bortezomib-dexamethasone (VRD) versus two-drug regimens, thalidomide-dexamethasone (TD), lenalidomide-dexamethasone (RD), or bortezomib-dexamethasone (VD) although the impact on OS has not been established until recently. In the United States the most commonly used induction regimens are the combination of RVD or CyBorD [47].

Carfilzomib-based combinations are emerging as some of the most potent induction regimens in MM. Carfilzomib has been combined with lenalidomide-dexamethasone (CRD), and in 53 newly diagnosed patients with MM, 38% achieved at least a near-complete response (nCR) after four cycles [27]. Stem cells were successfully collected in 34/35 patients. CRD was well tolerated with minimal neuropathy, allowing for prolonged administration with deeper responses over time. After a median of 24 cycles and 47.5 months of follow-up the 4-year PFS was 64% with a 93% OS rate [48]. Subsequently 76 patients received the same induction for four cycles and underwent autologous HCT after high-dose melphalan consolidation followed by CRD consolidation and prolonged maintenance. Median age was 59 years (range 40–76), with 57% of patients with ISS stage II/III and 36% with high-risk cytogenetics. Response rates after four cycles of CRD consolidation post-auto-HCT were 96% VGPR, 73% CR, and 69% sCR. Among CR patients tested 82% of them were MRD negative by flow and 66% by next-generation sequencing (NGS). With a median follow-up of 17.5 months 2-year PFS was 97% and 2-year OS was 99% for all 76 patients. These excellent results need to be confirmed and compared head to head with bortezomib, lenalidomide, and dexamethasone before CRD can be considered standard of care. CRD was well tolerated with few grade 3 or 4 toxicities generally lymphopenia (28%), neutropenia (18%), and infections (8%). Only two patients were reported to have an asymptomatic decrease of ejection fraction to 45–50%.

Timing of HCT

The achievement of major responses (CR and VGPRs) to induction therapy with IMIDs and proteasome inhibitors, especially in combination, has called into question the role of HDT and HCT as consolidation of first remission for all patients with MM. Yet, even in the context of modern induction regimens where over 90% respond the quality of response continues to improve following HCT and PFS approaches or exceeds 3 years [15, 16]. Four large randomized trials have now been performed comparing early versus delayed HCT in the context of modern induction treatment and are summarized in Table 29.4.

Palumbo et al. randomized 273 newly diagnosed MM patients to receive an autologous HCT after melphalan at a

Table 29.4 Common toxicities associated with high-dose melphalan

Toxicity	Incidence	Time peak occurrence	Preventive measures
Cytopenias	100%	5–7 days post-HCT infusion	Filgrastim or Peg-filgrastim will reduce duration of neutropenia Transfusion support not universally required Cell dose of greater than 5×10^6 CD34+ cells not effective in reducing cytopenias
Mucositis	30%	5–7 days post-infusion	Cryotherapy effective in reducing incidence of severe mucositis Keratinocyte growth factor not effective nor indicated Incidence increases with prior exposure to cytotoxic chemotherapy
Infections	40–50%	During cytopenic fever	Neutropenic fever common Severe sepsis and life-threatening infections rare. HSV reactivation rare with acyclovir prophylaxis CMV reactivation rare (<5%), thus monitoring not indicated Fungal infection rare, but fluconazole prophylaxis during neutropenia required

dose of 200 mg/m^2 or continued treatment with melphalan-prednisone-lenalidomide (MPR) followed by a second randomization to lenalidomide maintenance therapy. After a median follow-up of 51.2 months the PFS was significantly longer with high-dose melphalan plus HCT compared to MPR (43.0 months vs. 22.4 months $P < 0.001$). Overall survival at 4 years was also superior for high-dose melphalan and auto-HCT (81.6% vs. 65.3%; $P = 0.02$). Median progression-free survival was significantly longer with lenalidomide maintenance than with no maintenance (41.9 months vs. 21.6 months; $P < 0.001$), but 3-year overall survival was not significantly prolonged (88.0% vs. 79.2%) [49].

Gay et al. recently reported on 389 newly diagnosed MM patients less than 65 years of age receiving induction with four 28-day cycles of lenalidomide (25 mg, days 1–21) and dexamethasone (40 mg, days 1, 8, 15, and 22). Patients were randomized to consolidation with either chemotherapy plus lenalidomide (six cycles of cyclophosphamide [300 mg/m^2, days 1, 8, and 15], dexamethasone [40 mg, days 1, 8, 15, and 22], and lenalidomide [25 mg, days 1–21]) or two courses of high-dose melphalan (200 mg/m^2) and auto-HCT with a second randomization to maintenance with lenalidomide (10 mg, days 1–21) plus prednisone (50 mg, every other day) or lenalidomide alone. With a median follow-up of 52 months PFS was significantly shorter with chemotherapy plus lenalidomide compared with high-dose melphalan and ASCT (median 28·6 months vs. 43·3 months $P < 0{\cdot}0001$). Fewer grade 3 or 4 adverse events were recorded with chemotherapy plus lenalidomide than with high-dose melphalan and HCT. At 4 years, overall survival was 86% for autologous HCT with lenalidomide maintenance vs. 73% with chemotherapy plus lenalidomide maintenance [50].

The Intergroup Francophone du Myeloma-Dana-Farber Cancer Institute (IFM-DFCI) study of bortezomib, lenalidomide, and dexamethasone with or without high-dose melphalan consolidation and HCT was presented in December of 2015 at the American Society of Hematology meeting. A total of 700 patients were randomized to receive either eight cycles of RVD followed by lenalidomide maintenance or three cycles of the same induction followed by high-dose melphalan consolidation and followed by two cycles of the same chemo as consolidation with subsequent lenalidomide maintenance. With a median follow-up of 39 months HCT improved the complete response rate (58% vs. 46%) and 3-year PFS from 48% in the delayed HCT arm to 61% in the early HCT arm ($P < 0.0002$). The PFS benefit was observed in all subgroups. The 3-year post-randomization OS rate of overall survival was 88% in both groups [51].

Cavo et al. recently updated the results of EMN02/HO95 MM Trial in which patients were randomized to either four 42-day cycles of bortezomib-melphalan-prednisone (VMP) vs. either a single course or two sequential courses of melphalan at 200 mg/m^2 (HDM) with autologous stem cell support. All patients received induction therapy with CyBorD for 3–4 cycles. A second randomization to consolidation therapy with RVD vs. no consolidation was performed after intensification, to be followed by lenalidomide maintenance until progression or toxicity in both arms. From February 2011 to April 2014, 1510 patients 65 years or less were registered. Of these, 1192 were randomly assigned to receive either VMP ($n = 497$ patients) or HDM (1 ± 2 courses) ($n = 695$ patients). Median age was 58 years in both groups, ISS stage III was 21% in VMP and 20% in HDM, while revised ISS stage III was 9% in both groups. With a median follow-up from registration of 26 months median PFS was 44 months in the VMP arm and was not yet reached in the HDM arm; 3-year estimates of PFS were 57.5% and 66%, respectively ($P = 0.003$). PFS benefit with HDM was retained across predefined subgroups, including patients with ISS stage, cytogenetic risk category. The probability of achieving a very good partial response or higher quality response was 85.5% in the HDM group vs. 74% in the VMP group ($P < 0.001$). In a multivariate Cox regression analysis stratified by ISS, randomization to HDM and absence of high-risk cytogenetic abnormalities were the most important independent predictors of prolonged PFS. No difference in OS has been seen among the groups but the limited follow-up and small numbers of events make any conclusions difficult at this time [52].

Role of Tandem Transplants

Two studies have suggested that two courses of high-dose melphalan are superior to a single SCT, but the benefit appears to be limited to patients who had not achieved VGPR after the first transplant [53, 54]. Neither of these studies included induction therapies with either an IMID or a proteasome inhibitor and therefore may not be relevant today. More recently, patients who received bortezomib-based induction therapy and either single or double SCT on European phase III trials were analyzed. In comparison with patients for whom a single SCT was planned by study design, those who were assigned to receive tandem SCT had significantly longer PFS (median: 38 vs. 50 months, $P < 0.001$) and OS (5-year estimates: 63% vs. 75%, $P = 0.002$). From this dataset, the benefit of tandem ASCT was greatest for patients with high-risk cytogenetics defined as t(4;14) and/or del 17p and also for those who had not attained CR following bortezomib-based induction [55].

More recently, Cavo et al. presented the first interim analysis of the European Myeloma Network 02 (EMN02). Six hundred and fourteen eligible patients who received the diagnosis of MM in centers with a double-intensification policy were randomly assigned to either VMP ($n = 199$) or to a single ($n = 208$) or a tandem ($n = 207$). With similar patient and disease characteristics and a median follow-up of

27 months the median PFS was 45 months in the single-HCT group and has not been reached in the tandem HCT group. Three-year PFS were 60% and 73%, respectively ($P = 0.030$). Tandem HCT was found to be associated with an increased PFS in all predefined subgroups. In a multivariate Cox regression analysis stratified by ISS stage, randomization to tandem HCT and high-risk cytogenetics (any of the five pre-specified abnormalities) were the leading independent predictors of PFS. No difference in OS was seen between the two treatment groups [56].

In contrast the Blood and Marrow Transplant Clinical Trials Network (BMT-CTN) StAMINA Trial randomized 758 patients to one of the three consolidation strategies after an initial melphalan 200 mg/m^2 autologous HCT. A third of the patients (n = 254) received four cycles of consolidation with RVD, another third (n = 247) underwent a second autograft also with melphalan 200 mg/m^2, and the last third (n = 257) received no further consolidation. All three groups received lenalidomide maintenance 5–15 mg daily. At 38 months post-randomization the PFS was 52% for lenalidomide maintenance, 57% for RVD consolidation, and 56% for tandem transplant. OS at 38 months was excellent in all three groups (83, 86, and 82%). It is important to note that in the BMT CTN trial 32% of patients randomized to the tandem transplant arm did not received their "per-protocol" therapy which could explain some of the differences with the HOVON EMN trial of Cavo et al. [57].

Role of Allogeneic HCT

Although a graft-versus-MM effect has been well documented allogeneic HCT as frontline therapy for MM should be limited to younger patients with very-high-risk features due to high treatment-related mortality (TRM) and the risk of graft-versus-host disease even with non-myeloablative regimens. The European Group for Blood and Marrow Transplantation Non-Myeloablative allogeneic stem cell transplantation in MM 2000 study compared tandem HCT and reduced-intensity conditioning (RIC) allogeneic transplantation to HCT (single or tandem optional) alone in 357 patients that were biologically randomized based on the availability of an HLA-identical sibling. At a median of 96 months of follow-up, PFS and OS were 22% and 49% vs. 12% (P = 0.027) and 36% (P = 0.030) with HCT/RIC allogeneic HCT and autologous HCT, respectively [58]. The largest study including 625 patients performed through the BMT-CTN biologically assigned patients to tandem ASCT or RIC allogeneic and showed that at 36 months of follow-up there was no difference in PFS or OS [59].

Two other prospective studies, from the HOVON and Italian Study groups with somewhat different trial designs, have failed to conclusively show a benefit for allogeneic HCT in the up-front treatment of MM despite some suggestion of a better outcome with RIC allogeneic SCT in the Italian study [60, 61]. However, the tandem HCT arm of that study has a curiously short survival (median 48 months). Recent registry data assessing the role of allogeneic HCT in over 1200 patients with MM reported 5-year PFS and OS of 14% and 29%, respectively, with older age, longer interval from diagnosis to transplantation, and unrelated donor grafts adversely affecting OS [62].

Role of Salvage HCT

With the increasing number of patients opting for delayed HCT the optimal treatment for patients relapsing after primary therapy that did not include HCT, re-induction treatment with combination chemotherapy is the standard and most experts agreed that high-dose therapy consolidation should be considered the standard of care for this patient population. Data from both the CIBMTR and the EBMT show increasing use of salvage HCT, but prospective trials are urgently needed in this setting [63].

Cook et al. reported the first prospective randomized trial studying autologous HCT versus less intensive alkylating agent consolidation (weekly cyclophosphamide). Patients were eligible if they had relapsed after an initial autologous HCT and had at least an 18-month remission. Re-induction therapy included bortezomib, doxorubicin, and dexamethasone induction therapy. Eligible patients (with adequate stem cell harvest) were randomly assigned (1:1) to either high-dose melphalan 200 mg/m^2 plus salvage HCT or oral cyclophosphamide (400 mg/m^2 per week for 12 weeks). Time to progression was longer for patients who underwent salvage HCT (19 vs. 11 months ($P < 0.0001$) [64].

Salvage allogeneic HCT is being routinely performed particularly in younger patients with either multiply relapsed MM or short initial remission after autologous HCT. Freytes et al. performed a large registry analysis comparing the outcomes of a second autotransplant to those of a salvage allograft with a reduced-intensity conditioning regimen. NRM at 1 year after transplantation was higher in the allograft group 13% vs. 2%. Three-year PFS and OS were 6% and 20% for the allograft group and inferior to the outcomes for the autologous HCT 12% and 46%, respectively. [65]. In contrast, Patriarca et al. analyzed outcomes in patients relapsing after an initial autograft. The 2-year NRM was 22% among the 75 patients who had an identified HLA-compatible donor (donor group) versus 1% for those without a donor (no-donor group). The 2-year PFS was 42% in the donor group and 18% in the no-donor group ($P < 0.0001$) with similar 2-year OS of 54% and 53% for the donor and no donor groups, respectively [66].

De Lavallade et al. compared the outcomes of 32 relapsed MM patients. Nineteen had an HLA-identical sibling donor ("donor" group), while 13 patients had no donor ("no-donor" group). There were no significant differences between these two groups as for prognosis risk factors. With a median follow-up of 36 months, OS was similar between both groups; however, PFS was significantly higher in the "donor" group as compared to the "no-donor" group (46 vs. 8% at 3 years) [67].

Role of Maintenance Therapy and Consolidation Post-HCT

Post-HCT consolidation therapy was initially reported by Ladetto et al. who showed that four cycles of VTD consolidation following tandem HCT in patients with MM who achieved at least a VGPR ($N = 39$) increased the frequency of CR from 15 to 49% and molecular remissions from 3 to 18% [68].

Post-HCT consolidation has traditionally included similar drugs given during the induction phase. Cavo et al. reported on 480 newly diagnosed MM patients who received induction with VTD or TD that was followed by tandem HCT and two cycles of VTD or TD consolidation according to the induction arm [69]. Following HCT, the CR rate was 61% after VTD and 47% after TD consolidation ($P = 0.012$). To date, PFS but not OS is longer in the VTD arm.

The Nordic Myeloma Study Group randomized 370 bortezomib-naïve patients to 20 weekly doses of bortezomib or no consolidation at 3 months post-transplantation [70]. Again, response rates improved in patients who received consolidation with 71% vs. 57%, ($P < 0.01$) achieving at least VGPR and PFS was extended (27 vs. 20 months, $P = 0.05$), but the advantage was only seen in patients with <VGPR after HCT. There was no difference in OS between the groups. In contrast, the BMT-CTN StAMINA trial did not show a benefit for RVD consolidation followed by lenalidomide maintenance when compared to lenalidomide alone [57].

An extensive review of the different strategies and drugs tried as maintenance therapy after autologous HCT is beyond the scope of this chapter. Table 29.5 summarizes the three randomized trials demonstrating a PFS benefit for lenalidomide maintenance. The role of lenalidomide maintenance post-autologous HCT is fairly established [71–74]. The CALGB 100104 study examined 462 MM patients who were randomized to lenalidomide or placebo without consolidation until disease progression [71]. A significant increase in TTP was seen for patients in the lenalidomide arm compared with those receiving placebo (46 vs. 27 months, $P < 0.0001$). All patients benefitted from lenalidomide maintenance regardless of remission status or prior exposure to IMID therapy; at 48 months of follow-up despite crossover of 71% of placebo patients, the risk of death on lenalidomide maintenance is lower than on placebo (20% vs. 30%; $P = 0.008$) [71].

Table 29.5 Lenalidomide maintenance (versus placebo) following ASCT

	CALGB 100104 [71]	IFM 2005-02 [72]	RV-MMP 1209 [73]
N	460	614	200 (randomized to HCT)
Initial dosing	10 mg (5–15 mg) daily	10 mg (5–15 mg) daily	10 mg (5–15) 21/28 days
Duration	Until progression	24 months	Until progression
TTP/PFS	TTP: 46 vs. 27 months ($P < 0.001$)	PFS: 41 vs. 23 months ($P < 0.001$)	PFS 54.7 vs. 37.4 months
OS	3-year OS: 88% vs. 80% ($P = 0.028$)	5-year OS: 68% vs. 67%	5-year OS 78.4% vs. 66.6%
SPM total	18 vs. 6 cases	23 vs. 8 cases	11 cases (5 in lenalidomide)

The IFM 2005–02 trial reported on 614 patients who were randomized to lenalidomide or placebo after single (79%) or tandem (21%) and two cycles of lenalidomide consolidation [72]. Lenalidomide maintenance improved median PFS (41 months vs. 23 months) compared to placebo ($P < 0.001$). However, the 5-year post-randomization OS is similar (68% vs. 67%). A higher incidence of secondary primary malignancies (SPM) in the lenalidomide arm was detected in both studies.

Palumbo et al. randomized 200 patients who had been randomized to receive high-dose melphalan consolidation to lenalidomide maintenance given 21 out of 28 days. Similar to the other two trials a significant benefit on PFS was seen in patients randomized to the lenalidomide arm (54 vs. 37 months from diagnosis) but without an increase in SPM [73].

A recent meta-analysis of the three large randomized trials was recently presented at the American Society of Clinical Oncology Meeting in Chicago in July 2016. A total of 1209 patients were randomized from 2005 to 2009 to receive either lenalidomide or placebo. With a median follow-up of 6.6 years the median OS for lenalidomide maintenance had not been reached vs. 86 months for patients in the control arms ($p = 0.001$). All patients benefitted from lenalidomide maintenance regardless of response [74].

Future Directions

Despite doubling of the remission duration with post-autologous HCT lenalidomide maintenance, MM recurrence remains the most important cause of treatment failure. Depth of response has been shown to impact remission duration and survival; notwithstanding, even patients achieving a CR will relapse and progress [75, 76].

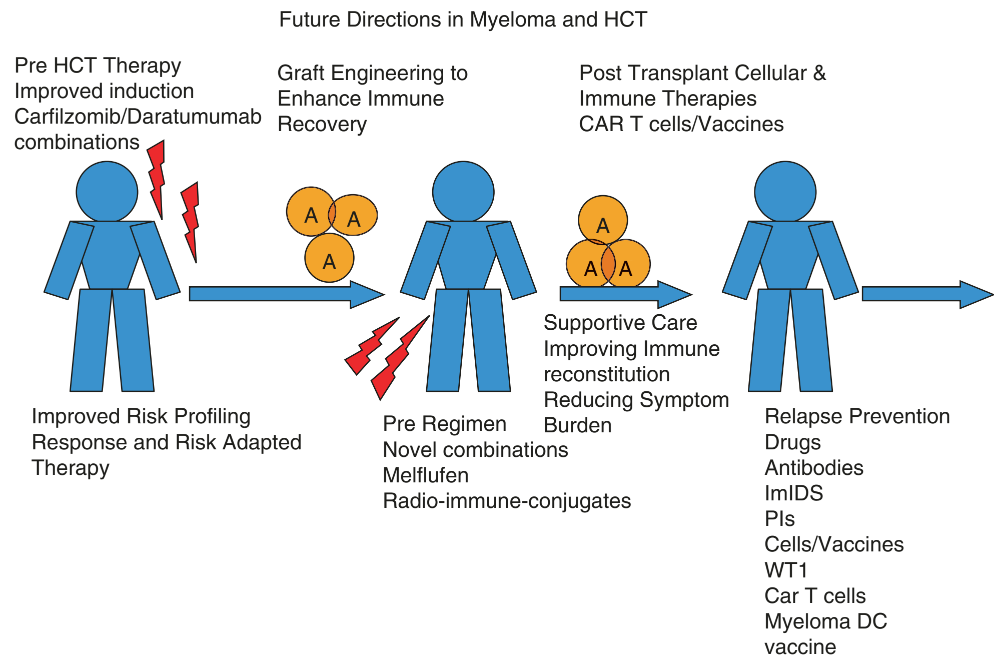

Fig. 29.1 Future directions in myeloma hematopoietic cell transplantation

Minimal residual disease detection (MRD) either by multiparameter flow cytometry (MFC) or next-generation sequencing (NGS) has been shown to be an important marker for long-term disease control in patients achieving a CR (reviewed in [77]).

Thus strategies aimed at increasing the proportion of patients that can attain an MRD-negative state are being actively explored and are summarized in Fig. 29.1. These include novel induction regimens incorporating carfilzomib and/or daratumumab as well as novel posttransplant immunotherapies such as CAR T cells, vaccines, and checkpoint blockade [78–81]. Likewise, incorporation of MRD assessment as part of a risk-adapted strategy was considered a “high-priority area to study” at the recent State of the Science Symposium of the BMT-CTN and will probably dictate treatment paradigms in the future [81–83].

As molecular techniques evolve, the goal is to individualize therapy based on predictive markers that provide insight into the likelihood of response and/or toxicity to certain drugs or regimens. Robust data already exists regarding the benefit of proteosome inhibition in the treatment of high-risk MM [84]. More recently, venetoclax a BCL 2 inhibitor has been shown to be extremely active in MM patients with the 11,14 translocation [85]. Thus the era of targeted therapies for specific MM subtypes has begun.

Finally, the burden of treatment both in regard to symptom burden cost of therapy will become a major issue in MM therapy and will be important endpoints to consider when choosing the treatment strategy that will provide patients with the longest life and the best quality of life with the least treatment burden.

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30 Solitary Plasmacytomas and Soft-Tissue Involvement in Multiple Myeloma

Joan Bladé and Laura Rosiñol

Localized Plasmacytomas

Localized plasmacytomas are plasma cell tumors, histologically indistinguishable on multiple myeloma that develop as single tumors either in bone (solitary plasmacytoma of bone—SPB) or in soft tissues (extramedullary—EMP) [1]. Both are uncommon disorders accounting for less than 5% of all plasma cell malignancies [2–15].

Solitary Plasmacytoma of Bone

Clinical Findings and Diagnostic Criteria

Solitary plasmacytoma of bone (SPB) was first recognized almost one century ago and since then single-case reports as well as a number of series have been reported [2–15]. The diagnostic requirements have been revised by the International Myeloma Working Group [1]. SPB consists of a single plasma cell tumor localized in bone and it is an uncommon disorder with a frequency ranging from 3 to 5% of all plasma cell neoplasms. Typically the histopathological pattern shows a diffuse involvement by mature plasma cells identical to those observed in multiple myeloma (MM). SPB is more frequent in males than in females (ratio 2:1) and the median age is about 10 years less than that observed in patients with MM. The most frequent complain at presentation is pain at the site of the skeletal lesion. The bones more commonly involved are vertebrae followed by sternum, pelvis, and proximal long bones. Thoracic vertebrae are more commonly involved than lumbar and cervical. Back pain or features of spinal cord compression are the most frequent presenting features. Soft tissue from a plasmacytoma, as in the sternum, may result in a palpable mass. The diagnostic criteria are shown in Table 30.1. To ensure that there are no other plasmacytomas radiological imaging techniques are necessary. Classically, it was required that a complete skeletal survey did not show other lesions. Today the skeletal survey must be completed with a PET/CT to exclude other lesions. The bone marrow aspirate must contain less than 10% bone marrow plasma cells. Theoretically, there should be no serum or urine M-protein. However, more than 50% of the patients have a small M-component. Of course, it should be no evidence of CRAB (i.e., hypercalcemia, renal function impairment, or anemia related to the plasma cell proliferation) features apart from those resulting from localized bony plasmacytoma [1].

Table 30.1 Solitary plasmacytoma of bone: diagnostic criteria

Criteria
– Single area of bone destruction due to the plasma cell proliferation
– Bone marrow with <10% plasma cells
– Absence of other skeletal lesions on PET/CT
– No anemia, hypercalcemia, or renal impairment
– Absence of serum and urine M-protein[a]

[a]50% of patients have a small serum M-component

Prognostic Features

The prognosis of SPB depends on the risk of transformation to MM since progression to MM is observed in up to 75% of cases. The median time to progression is 2–4 years and the median overall survival ranges from 8 to 12 years [2–15]. The most frequent features associated with progression to MM are (1) involvement of the axial skeleton versus long bones, (2) older age, (3) persistence of the M-protein after treatment with radiation therapy, (4) plasmacytoma size larger than 5 cm, (5) low levels of uninvolved immunoglobulins, (6) abnormal free light chain (FLC) ratio at diagnosis, (7) presence of focal lesions at magnetic resonance imaging (MRI) or more than one

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Table 30.2 Solitary plasmacytoma of bone. Adverse prognostic features

– Older age
– Involvement of axial skeletal versus long bones
– Plasmacytoma size ≥5 cm
– Immunoparesis
– Persistence of the M-protein after radiation therapy
– Abnormal free light chain ratio
– Presence of focal lesions on MRI or >1 metabolic lesion at PET
– Abnormal bone marrow flow cytometry

metabolic lesion at PET examination, and (8) abnormal bone marrow flow cytometry (Table 30.2). In a recent study, only immunoparesis emerged as negative predictor for progression to MM at the multivariate analysis [16]. In this study, classical prognostic factors such as age, disappearance of the M-protein after radiation therapy, location, or tumor size did not have impact on outcome. In an MD Anderson series of 60 patients, 45 (75%) had a serum M-protein at diagnosis. Most of the 32 in whom the M-protein persisted after radiation therapy evolved to MM while only 1 of the 13 patients in whom the M-protein disappeared with therapy developed MM [17]. Of note, in all these 13 patients the M-protein disappeared within the first year beyond radiation therapy was finalized and 7 of them were free from progression to myeloma between 16 and 25 years of follow-up [17]. The Mayo Clinic group reported the impact of serum FLC on the transformation rate to MM in a series of 116 patients diagnosed between 1960 and 1995 with SPB [18]. Forty-three patients progressed to MM after a median of 1.8 years. Fifty-four patients had an abnormal sFLC ratio and the progression rate at 5 years was 44% in patients with an abnormal sFLC ratio and 26% for those with normal sFLC ratio. The abnormal sFLC ratio at diagnosis had an adverse effect on both time to progression to MM and overall survival. The persistence beyond 1–2 years from diagnosis of a serum M-protein value of 5 g/L or higher was an additional factor associated with progression. Combining the FLC ratio at diagnosis and an M-protein of less than 5 g/L at 1 or 2 years beyond diagnosis a simple staging system with low (no features), intermediate (one risk factor), and high risk (both risk factors) was developed showing a 13, 26, and 62% rate of progression to MM at 5 years of follow-up [18]. In a recent study involving 43 patients, 48% had abnormal involved sFLC value and 64% an abnormal sFLC ratio while 33% had two or more hypermetabolic lesions at the PET/CT examination [19]. In a multivariate analysis, abnormal involved sFLC, and presence of at least two hypermetabolic lesions on PET/CT were the two predictors for early evolution to MM [19]. Finally, multiparameter flow cytometry (MFC) has recently been shown as a valuable biomarker to predict both high risk and low risk of progression. In this regard, Paiva et al. [20] studied 35 patients with SPB and 29 with EMP through MFC. Bone marrow clonal PCs were observed in 17 of the 35 (49%) patients with SPB and in 11 of the 29 (38%) of patients with EMP. Seventy-one percent of patients with positive flow versus only 8% of flow-negative SPB evolved to MM with a median time to progression of 26 months. In contrast, no significant differences in progression depending on the MFC findings were observed among patients with EMP. Of interest, MFC may also help to identify the so-called true SP characterized by flow-negative bone marrow and the absence of M-protein with an exceedingly rare rate of progression to MM [20]. Furthermore, almost identical results with MFC were reported by Hill et al. [21], in 50 patients with SPB. Aberrant PC phenotype was observed in 34 patients (68%). Progression to MM was observed in 72% of flow-positive patients versus in 12.5% for those flow negative, with a median time to progression of 26 months. In this study, the presence of urinary light chains was also highly predictive of the outcome with a progression rate of 91% for those with urine light-chain protein excretion versus 44% for those without. Using both parameters the authors identified a subset of patients (flow negative, no urine light chains) with a probability of progression as low as 7.7% and a high-risk subset with a bone marrow-positive flow and/or urine light-chain protein excretion with a progression rate of 75%. It is very likely that the patients with a bone marrow-positive MFC have in fact an "early myeloma" rather than a true SPB. In summary, the current most important predictors of progression to MM in patients with SPB are bone marrow MFC, serum FLC values, and finding of more than one hypermetabolic lesions on PET imaging [18–21].

Treatment Approach

In many instances patients have had surgery, with complete or partial tumor removal, as part of the diagnostic procedure. Apart from the diagnostic approach, the indications for surgery are internal fixation of fractures or as prevention of fractures, urgent decompressive laminectomy, or stabilization of spine using Harrington rods.

The treatment of choice is local radiation therapy involving the entire tumor volume with a margin of health tissue of at least 2 cm [2, 16, 22–24]. In case of vertebral plasmacytomas the irradiation field should include the proximal and distal uninvolved vertebrae. The recommended dose is between 40 and 50 Gy fractionated in over 4–5 weeks. It has been recommended that in plasmacytomas larger than 5 cm the radiation dose should be 50 Gy. However, the relationship between radiation therapy dose and tumor response is controversial when the radiation dose is higher than 35 Gy

[12, 13, 17, 23], since no clear relationship between the doses of local radiation therapy and the progression to MM has been clearly shown. It is considered that in the vast majority of cases adjuvant chemotherapy is of no benefit. The Greek group reported no improvement using either conventional chemotherapy or novel agents including bortezomib [16]. However, in this retrospective study, it might be a bias (i.e., poorer prognosis for the subset given adjuvant therapy versus radiation alone). In any event, the authors of this chapter strongly believe that in nonresponders to radiation therapy as well as in patients with bulky masses (i.e., >5 cm) located in pelvis, sternum, or a large bone such as humera or femora a multiple myeloma treatment approach should be considered, including high-dose melphalan followed by autologous hematopoietic stem cell rescue for younger patients. Currently, the response should be assessed by PET/CT imaging at least 3 months after radiation therapy is completed. For complete response all metabolic activity suggestive of active disease must have disappeared.

Multiple Solitary Plasmacytomas, Recurrent Plasmacytomas, and Macrofocal Myeloma

Among all patients diagnosed with "SPB," about 5% have more than one bone lesion with or without soft-tissue involvement and with no features consistent with MM [1]. Although patients with two or more "solitary" plasmacytomas can be treated with radiation therapy alone, we would favor a multiple myeloma treatment approach. In case of early recurrence (i.e., <2 years) either as a new lesion or as a local recurrence in an irradiated area a MM treatment approach is mandatory. In case of late recurrence (>2 years) as a new single lesion rechallenge with radiation therapy is reasonable.

The Greek group reported a variant of MM characterized by multiple lytic lesions with or without soft-tissue masses, less than 10% bone marrow plasma cells, and small M-protein in younger patients and associated with a favorable outcome [25]. This form of multiple myeloma had been previously recognized in patients younger than 30 years [26, 27]. The treatment approach is the same as for general multiple myeloma, including high-dose melphalan followed by autologous stem cell rescue whenever possible.

Extramedullary Plasmacytoma

Extramedullary plasmacytoma is an uncommon plasma cell disorder consisting of a plasma cell soft-tissue tumor. EMP may originate in many anatomical sites, although more than 90% developed in the head or neck area, particularly in the upper respiratory structures [2–4].

Clinical Findings and Diagnostic Criteria

The incidence of EMP is about 3% of all plasma cell malignancies. It is more frequent in males than in females (2:1) and the median age at diagnosis is 60 years [2–4]. The clinical features depend on the site and organ involved. As a result of the frequent locations in the upper respiratory tract patients usually present with symptoms such as nasal obstruction or discharge, epistaxis, hoarseness, or hemoptysis. Pain and tenderness at the plasmacytoma site may occur. EMP can develop in any organ including gastrointestinal tract, brain, thyroid, breast, testes, or lymph nodes [2, 4–6]. There is a predominance of IgA immunoglobulin type. The diagnosis is based on the finding of a plasma cell proliferation in an extramedullary site in the absence of MM (Table 30.3).

Treatment and Outcome

As in SPB, the treatment consists of fractionated radiation therapy at the total dose of 40–50 Gy over 4–5 weeks. Radiation therapy on local lymph nodes may be considered since up to 25% of patients with EMP of the head and neck may develop lymph node involvement. EMPs localized in the upper respiratory tract have a better outcome than those arising outside the head and neck area. Involvement of the adjacent bone has been reported as an adverse factor. Local relapses, including lymph node involvement, occur in up to 15% of cases. Progression to MM is uncommon with a reported frequency ranging from 8 to 30% [3, 4, 6–8, 10, 14, 16, 24]. The most prominent features of SPB and EMP are shown in Table 30.4.

Table 30.3 Extramedullary plasmacytoma: diagnostic criteria

– Single extramedullary tumor of clonal plasma cells
– Normal bone marrow
– Absence of other lesions on PET/CT
– No anemia, hypercalcemia, or renal impairment
– Absence of serum and urine M-protein[a]

[a]Some patients may have a small serum M-component

Table 30.4. Clinical features

	SBP	EMP
Median age (years)	55	55
M:F	2:1	3:1
Main location	Axial skeleton (vertebral)	Head and neck
M-protein (%)	50	<25
Progression to MM (%)	≥75	8–30
10-year survival (%)	40–50	70

Solitary plasmacytoma of bone (SPB) versus extramedullary plasmacytoma (EMP)

Soft-Tissue Plasmacytomas in Multiple Myeloma

Multiple myeloma (MM) is characterized by a proliferation of plasma cells (PCs) with a strong dependence on the bone marrow (BM) microenvironment. However, in up to one-third of the patients the plasma cells escape the microenvironment influences resulting in soft-tissue plasmacytomas [28]. The existence of soft-tissue involvement in MM is long-term known and old autopsy studies have shown an extra-skeletal involvement in up to 70% of patients [29–32]. In patients with multiple myeloma, the soft-tissue masses can have two different origins: (1) direct growth from skeletal lesions by disrupting the cortical bone and (2) plasma cell tumors resulting from hematogenous spread. The mechanisms involved in the extramedullary myeloma dissemination are not well understood. However, possible explanations are (1) decreased expression of adhesion molecules, (2) low expression of some cytokine receptors or downregulation of CXCR4 and its ligands, and/or (3) increased angiogenesis [33–42]. It is likely that the physiopathologic mechanisms of the two variants of plasmacytomas (hematogenous spread versus direct growth from lytic lesions) are different. Hopefully, future studies matching and comparing the characteristics of malignant BMPCs with those growing at soft tissues help to better understand the mechanisms of myeloma dissemination outside the bone marrow.

Definition

Pasmantier and Azar reported in 1969 the findings of 57 autopsy cases and proposed a classification in three stages according to the presence or absence of macroscopic tumor outside bones [43]. Stage I or intraskeletal: disease confined to the bone marrow or bone, stage II or paraskeletal: presence of soft-tissue masses arising directly from bones extending to paraskeletal areas, and stage III or extraskeletal resulting from metastatic or hematogenous spread. Of interest, in most patients in stage I and II the plasma cells were well differentiated (plasmacytic myeloma) whereas in the majority of patients in stage III the plasma cells were poorly differentiated (plasmablastic myeloma). However, the definition of extramedullary involvement in MM has not been uniform. Recently, the International Myeloma Working Group (IMWG) agreed on that two different types must be considered. (1) paraskeletal consisting of soft-tissue masses arising from skeletal lesions and (2) pure extramedullary resulting from hematogenous spread and involving only soft tissues with no contact with bone (Rosiñol et al., manuscript in preparation). Some patients develop simultaneously or successively the two types of plasmacytomas.

Incidence and Location

The reported incidence of paraskeletal plasmacytomas at diagnosis is from 7 to 34.4% [28, 44–48] while the reported rate of pure extramedullary involvement ranges between 1.7 and 4.5% [49, 50]. At relapse, the incidence of paraskeletal involvement remains similar to that observed at the time of diagnosis [44–48] while the frequency of extramedullary disease increases ranging up to 3.4–10% [49–53]. In two recent studies, 45 and 56% of patients with plasmacytomas at the time of diagnosis had paraskeletal or extramedullary disease at relapse [45, 54]. It has been suggested that patients undergoing allogeneic transplantation, particularly with dose-reduced intensity conditioning and those treated with novel antimyeloma agents, such as thalidomide, bortezomib, or lenalidomide, may have a higher incidence of plasmacytomas [55–57]. However, there is no evidence that the incidence of plasmacytomas increases at relapse after allogeneic transplantation or after exposure to novel antimyeloma agents [45, 48, 52, 54]. However, a better control of medullary disease with novel drugs can result in a more prolonged survival with a higher risk of extramedullary progression.

The extramedullary myeloma spread may consist of (1) single or multiple highly vascularized large red-purple subcutaneous nodules; (2) multiple small nodules located at any organ, particularly skin, liver, breast, or kidney; (3) pleura with myelomatous pleural effusion; (4) lymph nodes; and (5) central nervous system (CNS). Skin is the most frequent location at diagnosis whereas there is an increased incidence of liver, pleural, and CNS involvement at the time of relapse [49]. Leptomeningeal involvement occurs in about 1% of the patients [58–63]. The more common presenting features are confusion, paraparesis, and cranial nerve palsies. The cerebrospinal fluid (CSF) shows increased protein value and positive immunofixation for the myeloma M-protein as well as plasma cells usually with plasmablastic features. CNS involvement is associated with poor prognostic features such as high-risk cytogenetics, plasma cell leukemia, and high LDH serum levels. CNS involvement is usually seen in advanced phases of the disease along with the involvement of other extramedullary locations. However, in some instances CNS involvement can present at diagnosis or as isolated relapse in patients in complete remission. The prognosis is very poor with median survivals shorter than 3 months, even when novel agents are used [64]. Treatment of CNS involvement with intrathecal therapy with methotrexate, hydrocortisone, and cytosine arabinoside is unsatisfactory. Craniospinal radiation can be considered. It has been reported in a multicenter study that local plus systemic therapy can improve the prognosis [63].

Paraskeletal involvement is the most frequent cause of plasmacytomas in MM and, as previously mentioned, consists of soft-tissue masses arising from lytic skeletal

lesions [28]. The most common locations are vertebrae, ribs, sternum, skull, and pelvis. Plasmacytomas arising from vertebrae can cause spinal cord compression, the most common neurological complication of MM occurring in up to 10% of the cases [65]. The dorsal spine is the most frequently involved with a clinical picture consisting of back pain and paraparesis that can evolve to paraplegia in a matter of hours or days. The complication is a medical emergency that requires confirmation by an immediate MRI. Treatment with high-dose dexamethasone at a loading dose of 100 mg followed by 25 mg every 6 h with subsequent progressive tapering plus radiation therapy should be immediately started [66]. Plasmacytomas can be triggered by surgical invasive procedures usually performed during the course of the disease [67–70]. They can originate from laparotomy scars or catheter insertions and can even precede systemic relapses. Extensive extramedullary involvement resulting from bone surgery or fractures has also been reported [68].

Plasma Cell Characteristics in Soft-Tissue Plasmacytomas

Plasma cells from extramedullary disease commonly show immature or plasmablastic features. In contrast, myeloma cells from paraskeletal masses arising from focal bony lesions are less undifferentiated and show a more mature or plasmacytic morphology [28, 43]. CD56 expression is usually downregulated in plasma cells at extramedullary sites [71, 72]. However, more studies are needed to establish the role of CD56 in the extramedullary myeloma dissemination. The information on genetic abnormalities in extramedullary myeloma is limited. The frequency of high-risk cytogenetics, particularly 17p deletion, at extramedullary sites is usually higher than that reported in BMPCs [73–78]. It also seems that the hematogenous spread is more frequent in patients with gene expression profile (GEP)-defined high-risk myeloma [49]. However, molecular genetic studies on paired samples from medullary and extramedullary sites in the same patients are required in order to identify potential extramedullary disease-associated genes and to understand the mechanisms of extramedullary myeloma dissemination [49].

Assessment of Plasmacytomas

In some patients plasmacytomas consist of palpable masses which can be assessed by physical examination. However, in many instances radiographic imaging techniques are needed [79, 80]. Magnetic resonance imaging (MRI) is useful when spinal cord or nerve root compression is suspected and it is the best imaging technique to assess leptomeningeal or cerebral involvement [81]. The typical findings are leptomeningeal enhancement and/or meningeal based lesions resembling intracerebral masses [81]. Fluorodeoxyglucose (FDG) positron emission combined with computed tomography (PET/CT) is the most valuable whole-body technique in patients in whom the presence of plasmacytomas is suspected [82–86]. The main limitation of the PET/CT is that it is not standardized and the potential lack of interobserver reproducibility. A PET/CT should be done when myeloma soft-tissue involvement is suspected on the basis of clinical manifestations and in patients at high risk such as in those with high LDH serum levels as well as at the time of relapse in patients with previous history of plasmacytomas given the high frequency of plasmacytomas at relapse in this population [45, 54]. It is crucial and mandatory to include the plasmacytoma assessment when evaluating the response to therapy in patients with MM. The IMWG criteria requires the disappearance of plasmacytomas for CR and a decrease equal or higher to 50% for PR. Progression is defined by either the increase in at least 25% of preexisting masses, recurrence of a plasmacytoma that had disappeared with therapy, or development of any new soft-tissue involvement [87]. Ideally, the same imaging technique should be used at baseline and during follow-up [88].

Prognosis

The presence of soft-tissue involvement in MM is associated with a shorter survival. Thus, the Pavia group showed that the presence of soft-tissue involvement at any time during the course of the disease was associated to shorter PFS and OS [45] and the Royal Marsden group reported that the presence of plasmacytomas was associated to worse prognosis in patients treated with conventional chemotherapy [44]. Of interest, in the above two series [44, 45] as well as in a recent South Korea study [89] the administration of high-dose therapy followed by autologous stem cell rescue was able to overcome the negative impact of the presence of plasmacytomas. In contrast, in a PETHEMA transplant trial the OS of patients with paraskeletal involvement was significantly shorter in those with plasmacytomas [90]. It must be taken into account that in all the above studies the majority of patients had paraskeletal disease and only few pure extramedullary disease. In fact, the Arkansas group reported that patients with extramedullary disease had a significantly shorter PFS and OS even in the era of novel agents and treated in the context of the total therapy approaches [49]. Pour et al. [51] reported that in relapsed patients the presence of plasmacytomas was associated with a poorer outcome. Of interest, the survival of patients with extramedullary disease was significantly shorter than that of those with paraskeletal involvement [51]. The prognosis of patients with CNS disease is ominous even in the era of novel agents [64].

Treatment

In the frontline setting, alkylating agents, particularly high-dose melphalan, are of benefit in patients with paraskeletal disease while their efficacy in patients with hematogenous extramedullary disease is doubtful [28, 44, 45]. Bortezomib seems to be of benefit in patients with paraskeletal involvement with less evidence for hematogenous dissemination [91–93]. There is no published data on the efficacy of other proteasome inhibitors such as carfilzomib or ixazomib. The efficacy of IMIDs seems limited. In this regard, thalidomide is not effective in any type of plasmacytomas [94–98]. There is no published data on the efficacy of lenalidomide. The Mayo Clinic reported that 31% (4 out of 13) patients with extramedullary involvement responded to pomalidomide plus low-dose dexamethasone [50]. However, the authors of this chapter have treated nine patients with advanced MM and plasmacytomas with pomalidomide and low-dose dexamethasone and no response in plasmacytomas was observed. It is of interest that a dissociation between paraproteinemic and plasmacytoma response has been reported in patients treated with both thalidomide and bortezomib [93–96, 98]. The small sample size and the absence of controlled studies are important limitations to draw durable conclusions on the efficacy of bortezomib and IMiDs on soft-tissue involvement in MM. There are no reports on the potential efficacy of monoclonal antibodies such as elotuzumab and daratumumab on paraskeletal or extramedullary myeloma.

Considering that alkylating agents and bortezomib seem the most effective agents in patients with soft-tissue masses, although the evidence is lower for extramedullary than for paraskeletal disease, the treatment of choice for patients non-eligible for ASCT would be a combination of melphalan and prednisone with bortezomib (MPV) [99, 100]. Taking into account that high-dose therapy, particularly high-dose melphalan, can overcome the poor prognosis of paraskeletal involvement, a triple bortezomib-based regimen (VTD, PAD, or VRD) followed by ASCT could be the treatment of choice for younger patients [101, 102]. For transplant-eligible patients with extramedullary disease a combined anti-myeloma/anti-lymphoma regimen such as VTD/PACE followed by an allogeneic stem cell transplantation in patients younger than 50 years or by a tandem ASCR followed by dose-reduced intensity conditioning allogeneic transplantation (Allo-RIC) in those aged 50–65 years should be considered [103].

The prognosis of patients with MM relapsing with soft-tissue involvement (i.e., paraskeletal or extramedullary) is very poor [48–53]. Since these patients have already received previous bortezomib and/or IMiD-based regimens, the most effective treatment consists of lymphoma-like regimens such as PACE, Dexa-BEAM, or HyperCVAD [28, 104, 105]. The response rate is about 50%; however, the median duration of response is of only 4 months [105]. For this reason, in patients who are eligible for ASCT the best approach would be the administration of two or three cycles immediately followed by the high-dose procedure.

Local radiation therapy should be urgently administered in case of spinal cord compression and also considered in patients with severe compressive pain, bulky plasmacytomas, and persistent local disease after systemic therapy. There are not yet data on the potential efficacy of monoclonal antibodies such as elotuzumab or daratumumab, the new proteasome inhibitors carfilzomib and ixazomib, or the new IMiDs or other drugs still in their early development.

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31 Supportive Care in Multiple Myeloma

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Myeloma Bone Disease

Multiple myeloma (MM) is the second most common hematologic malignancy, affecting more than 60,000 patients in the United States with 30,000 patients diagnosed in 2016 [1, 2]. Multiple myeloma (MM) is the most frequent cancer to involve bone with almost 70% of patients presenting with bone lesions at diagnosis [3]. Twenty percent of patients present with a pathologic fracture at diagnosis and until recently up to 60% of patients developed a pathologic fracture over the course of their disease [7]. This is extremely important since pathologic fractures increase mortality of MM patients by 20% [8]. In addition, MM bone disease can cause excruciating bone pain that remains undertreated in many patients, and hypercalcemia that can be life threatening, as well as require surgery and/or radiation to bone to control bone pain, treat impending or repair fractures, and relieve spinal cord compression. Further, in the vast majority of patients, MM bone lesions rarely heal even when patients are in long-term remission [10], and MM patients continue to suffer from the sequelae of their bone disease, even when their MM is under excellent control. Tremendous progress has been made in the treatment of MM, with a median survival of patients increasing to more than 6.1 years in the last decade and a much better overall survival for patients over the age of 65 [9]. Thus, treatment and repair of MM bone disease are increasingly important, both for enhancing the quality of life of patients, increasing their survival, prevention of hypercalcemia or fracture, and suppressing the growth of MM cells in the bone marrow.

Pathophysiology of Myeloma Bone Disease

During the normal bone-remodeling process, osteoclastic bone resorption is coupled to osteoblastic bone formation, so that bone removal and formation are balanced (Fig. 31.1). However, in MM the normal bone-remodeling process is essentially uncoupled, with markedly increased bone resorption at sites of MM and absent or severely decreased bone formation. This is due to suppression of osteoblast differentiation that can persist even when patients are in long-term remission [10]. Thus, little or no repair of lytic lesions occurs in the vast majority of patients with MM, although there have been anecdotal reports of healing of bone lesions in patients receiving bortezomib-based therapy. However, once a patient's bone density is below the fracture threshold, the patient continues to be at an increased risk of fracture and experience persistent bone pain.

A multiplicity of osteoclast stimulating factors are produced by MM cells and induced by MM cells in the bone marrow microenvironment [10]. These factors include MM cell production of Rank ligand, a highly potent osteoclast-stimulating factor, or induction of Rank ligand production by marrow stromal cells and osteocytes in the MM microenvironment. Furthermore, production of MIP-1 α by MM cells, interleukin 3 production by both MM cells and T-lymphocytes in the MM microenvironment, and IL-6 production by marrow stromal cells, osteoclasts, and other cells in the bone microenvironment also occur [10]. In addition, osteoclastic bone resorption and increased osteoclast numbers enhance the growth of MM cells. This process has been described as the vicious cycle hypothesis, in which MM cells induce osteoclastic bone resorption, and the bone resorption process releases growth factors, such as IGF1 and TGF-beta from bone matrix, which in turn stimulate the growth of MM cells (Fig. 31.2). In addition, MM cells induce polarization of T cells in the tumor microenvironment, which reverses the ratio of Th17 to Th1 T cells to 10:1 com-

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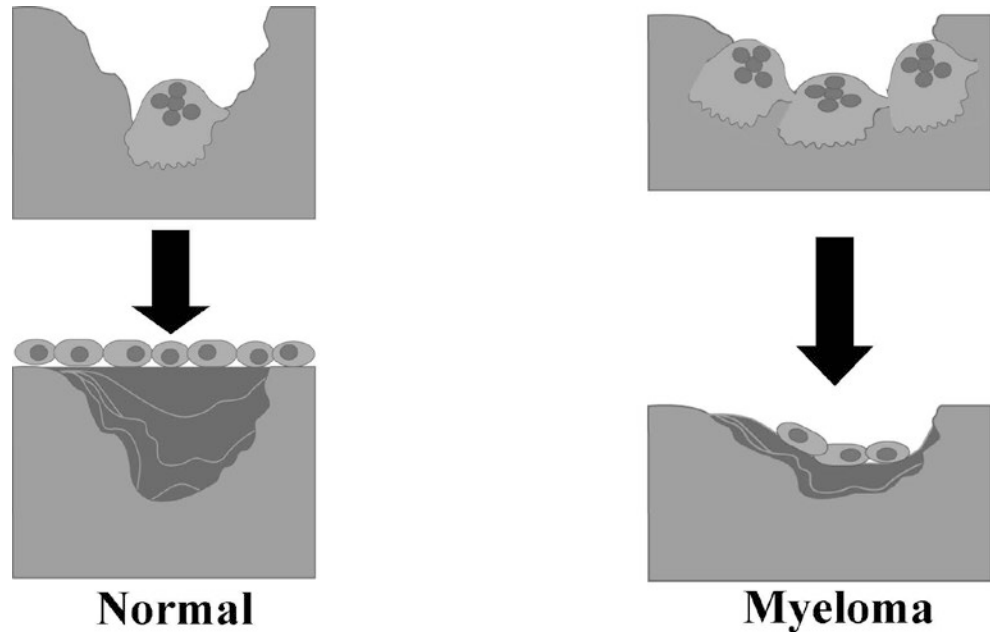

Fig. 31.1 Bone remodeling is uncoupled in myeloma. The normal bone-remodeling process in which bone resorption is followed by new bone at the same site is uncoupled in myeloma with increased bone resorption followed by little or no bone formation. Adapted by permission from Macmillan Publishers Ltd: Hattner R et al. Nature. 1965;206:489

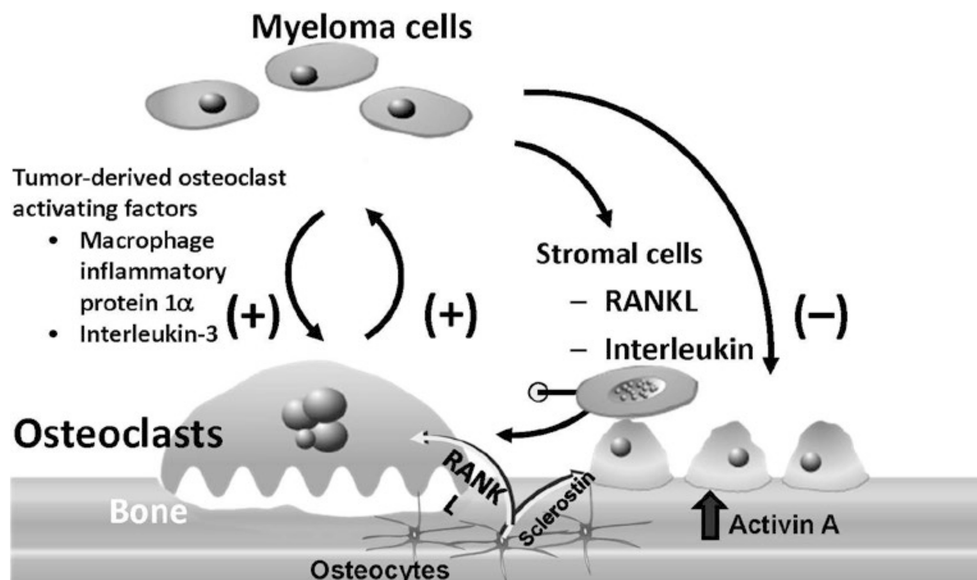

Fig. 31.2 Reciprocal interactions between myeloma cells and bone microenvironment contribute to progression of myeloma. Myeloma cells colonize the marrow and some of the cells engage the endosteal niche and become dormant, while other cells remain active myeloma cells. Active myeloma cells produce factors and induce factors by cells in the bone microenvironment to increase osteoclast formation and resorption. The increased bone resorption in turn releases growth factors from bone matrix to stimulate the growth of myeloma cells. Myeloma cells also suppress osteoblast differentiation to block new bone formation. In addition, myeloma cells directly interact with osteocytes in the bone matrix and these interactions increase myeloma cell growth and osteoclast formation as well as block osteoblast differentiation. Finally, osteoclastic resorption also releases dormant myeloma cells to become active myeloma cells, further contributing to myeloma progression. Adapted from Roodman GD. N Engl J Med. 2004;350(16):1655–1664. Copyright © 2004 Massachusetts Medical Society. Reprinted with permission

pared to the normal ratio of 1:10. Th17 cells produce IL17 that induces dendritic cell differentiation toward the osteoclast lineage, as well as enhance induction of Rank ligand.

MM cells, osteoclasts, and marrow stromal cells in the microenvironment also produce angiogenic factors that further increase the growth of MM cells [11]. In addition, the increased numbers of osteoclasts directly support MM growth. Yaccoby and coworkers demonstrated that primary MM cells from patients can be passaged over feeder layers of osteoclasts for long periods of time [12]. Abe and coworkers found that osteoclasts support the growth of primary MM cells through the production of IL-6 and osteopontin [13].

Finally, direct interactions between osteoclasts and osteocytes enhance bidirectional Notch signaling between the cells to increase the growth of MM cells, induce osteocyte apoptosis, and increase production of sclerostin and Rank ligand by osteocytes that suppresses osteoblast differentiation and stimulates osteoclastogenesis [14]. In addition, osteoclasts produce Annexin II, BAFF, and April that support MM cell growth [15, 16].

Recently, osteoclastic bone resorption has also been shown to play an important role in activating dormant MM cells in the MM microenvironment to active MM cells. Activation of dormant MM cells results in increased tumor burden, bone destruction, and distant colonization of bones. Lawson et al. recently showed by intravital imaging that treatment of mice with Rank ligand increases osteoclast numbers and decreases the numbers of dormant MM cells colonizing the osteoblast niche [17]. Thus, osteoclasts play multiple roles in progression of MM, including activation of dormant MM cells, bone destruction that results in release of matrix-bound growth factors to stimulate tumor growth, and enhancement of MM growth by factors derived from osteoclasts.

Studies in preclinical models of MM found that blocking osteoclastic bone resorption decreases tumor burden in addition to bone destruction [18]. However, until recently this phenomenon has not been demonstrated in patients. The MM IX trial showed that treating newly diagnosed MM patients with zoledronate, a potent bisphosphonate that blocks osteoclastic bone resorption, rather than with clodronate, a weaker bisphosphonate, enhances survival of MM patients. This increase in survival occurred regardless if the patients had detectable bone disease at diagnosis [19]. However, the greatest benefit in survival was seen in patients with detectable bone disease. Based on preclinical and clinical studies, the International Myeloma Working Group (IMWG) guidelines now support targeting osteoclastic bone resorption in all patients with MM [20].

Osteoblast Suppression in Myeloma

As noted above, bone formation is markedly suppressed in patients with MM and persists even when the MM is under excellent control. This explains why bone scans, which measure reactive bone formation, frequently under-represent bone disease in MM patients, making bone scans inappropriate for imaging bone disease in MM patients [21]. Multiple factors produced by MM cells or induced by MM cells in the MM microenvironment block osteoblast precursor differentiation to mature osteoblasts. A large number of inhibitors of osteoblast differentiation have been identified in MM patients, including TNFα, MIP-1 α, IL-3, activin A, sclerostin, DKK1, hepatocyte growth factor, TGF-beta, and IL7 [22]. All these factors can directly suppress osteoblast differentiation and are potential therapeutic targets for treating MM bone disease (see below). However, none of these factors can explain why osteoblast suppression persists in patients even in the absence of MM cells. The mechanisms responsible for the long-term suppression of osteoblast differentiation in MM are just beginning to be understood. D'Souza and all demonstrated that epigenetic changes in the Runx2 promoter in pre-osteoblasts, the master gene controlling osteoblast differentiation, are induced by MM cells [23]. These changes in the Runx2 promoter result from induction of Gfi1 in pre-osteoblastic marrow stromal cells exposed to MM cells. Gfi1 acts as a transcriptional repressor of Runx2 and blocks osteoblast differentiation. Increased expression of Gfi1 persists in marrow stromal cells from MM patients, even when the stromal cells are passaged for long periods of time in the absence of MM cells. Furthermore, knockdown of Gfi1 in marrow stromal cells from MM patients allows them to undergo osteoblast differentiation. These insights into the mechanisms responsible for long-term suppression of osteoblast differentiation have provided potential new therapeutic targets for repairing bone lesions in MM patients. Furthermore, Li and coworkers reported that mature osteoblasts could suppress MM cell growth through the production of decorin [24]. These results suggest that in addition to repairing bone lesions, inducing osteoblast differentiation may also suppress the growth of MM cells in patients.

Imaging of Myeloma Bone Disease

Imaging of MM bone disease has become increasingly important with the development of new criteria for the diagnosis of active MM. These criteria include presence of at least one lytic lesion detected by conventional radiography, whole-body low-dose CT or PET/CT, or presence of more than one focal bone marrow lesion greater than or equal to 5 mm on MRI studies [25]. Skeletal surveys have been the gold standard for detecting MM bone disease. The lesions appear as punched-out lytic bone lesions with the absence of reactive new bone formation and can occur in any bone. However, conventional radiographs are relatively insensitive for detecting bone disease in MM, since more than 30% of bone must be removed before a lesion is detectable. Conventional radiographs are also not useful for following disease progression or response to therapy, because lytic lesions rarely heal in these patients and enumerating new lesions can frequently be difficult in patients with extensive bone disease. In addition, it is often difficult to distinguish between osteoporotic and MM-induced vertebral fractures. Finally, skeletal surveys take a great deal of time to perform, which can be difficult for patients who have severe bone pain.

Low-dose whole-body CT has been recently shown to be superior to conventional radiographs for detecting osteolytic lesions in MM patients, and is especially useful for detecting lesions in the spine or pelvis [26]. Low-dose whole-body CT

can take less than 5 min to perform, and has the advantage that it can also demonstrate extraosseous lesions. Because of these advantages, low-dose whole-body CT is becoming the standard screening method for MM bone disease in Europe. However, although low-dose whole-body CT exposes the patients to a higher dose of radiation than standard skeletal surveys, the higher resolution and speed of the examination outweigh the disadvantages. Many experts predict that low-dose whole-body CT will replace skeletal surveys as the new gold standard for detecting MM bone disease [26].

Magnetic resonance imaging (MRI) is also more sensitive than standard skeletal radiographs for detecting MM bone disease, and also detects bone marrow infiltration by MM cells. A large study from the University of Arkansas showed that MRI detected focal lesions in 74% of MM patients, compared to 56% of patients who were examined with whole-body radiographs [27]. MRI is particularly useful for distinguishing between asymptomatic smoldering MM and symptomatic MM. Patients who were thought to have smoldering MM but had more than one focal lesion on MRI had a 70% probability of progression to active MM within 2 years [28]. These results form the basis for adding the presence of more than one focal criteria on MRI to the updated criteria for active MM [25]. The International Myeloma Working Group recommends, at a minimum, MRI examination of the spine and pelvis as part of the evaluation for smoldering MM, if whole-body MRI cannot be done [29]. However, MRI limited to the spine and pelvis will not detect lesions in the peripheral skeleton that occur in 10% of patients who present with focal lesions [30].

PET/CT is a very useful albeit expensive imaging technique for assessing MM bone disease. PET detects the hypermetabolic activity of MM cells in both intraosseous and extramedullary sites, while CT detects bone-destructive lesions. PET/CT has a similar sensitivity to that of MRI, and is better than MRI at detecting bony lesions. More importantly, PET/CT can be used to assess response to therapy [31]. Further, the presence of extramedullary lesions or an SUV Max greater than 4.21 on a PET/CT performed at diagnosis, persistence of FDG uptake following autologous stem cell transplantation for MM, or detection of extramedullary disease are all associated with a poor prognosis for patients. However, it is unclear if PET/CT or MRI is better for determining if patients with solitary plasmacytoma have disease restricted to one site or multiple sites. PET/CT is also useful for following patients with non-secretory MM [32]. However, PET/CT may also detect false-positive findings in patients following radiotherapy or infections.

Treatment of MM Bone Disease

The landmark studies of Berenson and coworkers in 1996 demonstrated that the nitrogen- containing bisphosphonate, pamidronate, decreased skeletal related events (surgery to bone, fractures, radiation to bone, and spinal cord compression) and bone pain in patients with MM bone disease [33]. Pamidronate and zoledronic acid are the cornerstone of treatment for MM bone disease. Bisphosphonates are non-hydrolyzable pyrophosphate analogues that block osteoclastic bone resorption and inhibit osteoclast formation through their capacity to inhibit protein prenylation by inhibiting farnesyl diphosphate synthase required for normal osteoclast activity [34]. Pamidronate is usually administered as a 90 mg intravenous infusion over 2 h, while its more potent analogue, zoledronic acid, is administered as 4 mg intravenously over 15 min. Both bisphosphonates have similar efficacy for treating MM bone disease and are routinely given every 3–4 weeks. Bisphosphonates are very safe drugs and their major toxicity is their potential deleterious effects on renal function. Therefore, serum creatinine levels must be measured prior to infusion. If creatinine levels in patients receiving these agents increase more than 10% from their baseline values, pamidronate or zoledronic acid should be withheld until the serum creatinine returns to within 10% of the previous baseline value. In patients who present with impaired renal function, the infusion time for pamidronate can be increased or the dose of zoledronate can be reduced, since renal toxicity of bisphosphonates is related to peak dose [35]. In patients who present with severe renal failure, initial therapy with bortezomib-based regimens should be started prior to initiation of bisphosphonate therapy (see below).

Multiple organizations have developed guidelines for use of bisphosphonates in MM. Each of these guidelines state that bisphosphonates can be given intravenously every 3–4 weeks for patients with MM bone disease that is identified by plain radiographs or MRI. More recent IMWG recommendations for treatment of MM-related bone disease state that bisphosphonate therapy should be considered for all patients with MM, regardless of the presence of osteolytic bone disease by conventional radiography [20]. These recommendations are based on the results of the MM IX trial that compared clodronate to zoledronic acid therapy in more than 1000 newly diagnosed patients receiving treatment for MM (see above). This study found that patients receiving zoledronate achieved a 5-month survival benefit compared to patients receiving clodronate [19]. This survival advantage occurred regardless if the patient had demonstrable bone disease at diagnosis. A subsequent secondary analysis of this study showed that the major impact on survival occurred in patients with bone disease [36]. However, it's unclear how long patients should continue to receive bisphosphonate therapy. This question became a major concern with the recognition that some MM patients receiving bisphosphonates develop bisphosphonate-associated osteonecrosis of the jaw (BRONJ). This complication of bisphosphonate therapy was first recognized by Marx et al. [37]. The occurrence of

BRONJ is usually dependent on the duration and dose of bisphosphonate therapy, undergoing invasive dental procedures, age of the patient, and treatment with glucocorticoids. BRONJ occurs more frequently in patients receiving zoledronate than pamidronate [37]. Because of concern for development of BRONJ, most guidelines recommend treatment with bisphosphonates for approximately 2 years, unless active MM persists, and then stopping or increasing the interval between administration of bisphosphonates should be considered. However, with the regular institution of close dental monitoring, dental prophylaxis, and performing most dental procedures prior to initiating bisphosphonate therapy, the incidence of BRONJ has markedly decreased.

Another question that often occurs is should bisphosphonate therapy should be stopped prior to invasive dental procedures. The American Dental Association recently recommended not stopping bisphosphonates or other bone-targeted agents when patients undergo dental procedures, and states that the decision to withhold bisphosphonate therapy should be based primarily on the patient's risk of developing a skeletal related event [38]. In contrast, the IMWG recommends stopping bisphosphonates for 90 days before and after invasive dental procedures, such as tooth extraction, dental implants, and surgery to the jaw [20].

Several studies assessed if bone resorption markers are useful for determining the interval between bisphosphonate administration in patients with MM bone disease. Patel and coworkers found in patients who had received bisphosphonate therapy for at least a year and had stable disease that the level of bone resorption markers could be used to determine when to administer bisphosphonate therapy [39]. However, other studies show that bone resorption markers are not sufficiently sensitive to guide bisphosphonate therapy [40]. Importantly, the MM IX trial demonstrated that extending bisphosphonate treatment beyond 2 years continued to have beneficial effects on skeletal related events [36]. However, only small numbers of patients received long-term bisphosphonate therapy in that study. Another consideration with bisphosphonate therapy is whether bisphosphonate therapy can be restarted in patients who developed BRONJ. A retrospective analysis of over 100 MM patients demonstrated that about 50% of patients who developed BRONJ healed their lesions and 50% of those who restarted bisphosphonates did not develop new lesions [41]. These results suggest that patients who have active MM and have healed their osteonecrosis of the jaw can receive bisphosphonate therapy but should be monitored closely for a reoccurrence of BRONJ.

In addition to bisphosphonates, denosumab, a human monoclonal antibody that targets Rank ligand, has been developed, and potently inhibits osteoclast formation and bone resorption. Multiple large phase 3 studies of denosumab versus zoledronate treatment for patients with bone metastasis found that denosumab has either a non-inferior or a superior effect on skeletal related events compared with zoledronate, and patients treated with denosumab who had breast cancer bone metastasis had a survival advantage [42]. A large phase 3 study compared denosumab to zoledronic acid treatment in patients with bone metastasis that did not have prostate cancer or breast cancer, and also included 200 MM patients. This study found a similar decrease in skeletal related events in the MM patients with either treatment, but a post hoc analysis found a decreased survival for MM patients receiving denosumab [43]. Further analysis of this trial found that the baseline characteristics of the MM patients receiving zoledronic acid or denosumab were not balanced, and that the patients receiving zoledronic acid treatment had more early withdrawals from the study compared to those treated with denosumab [43]. A large phase 3 study comparing denosumab to zoledronic acid treatment in newly diagnosed patients with MM bone disease has just been completed, and the results of this trial should be reported soon. It is important to note that the incidence of ONJ is similar in patients receiving denosumab or zoledronic acid so that similar dental screening, routine dental prophylaxis, and follow-up are also required for patients receiving denosumab. Finally, neither denosumab nor zoledronic acid increases bone formation in patients with MM, so there is still a great need for bone anabolic agents that can increase bone formation and are safe for MM patients.

Recently, several agents have been developed to enhance bone formation in patients with MM. These include an antibody to DKK1, BHQ880, and sotatercept, an activin receptor antagonist [22]. A phase 1B clinical trial of anti-DKK1 in patients with MM did not show any benefit for MM bone disease [44], although the anti-DKK1 did show some bone anabolic effects in a phase 2 trial of patients with smoldering MM. No anti-MM activity was found in MM patients or smoldering MM patients receiving anti-DKK1 therapy. Sotatercept is in clinical trial for MM but can also increase hemoglobin levels significantly [45]. Thus, its utility as a bone anabolic agent of MM is still unclear. Most recently an anti-sclerostin antibody has been developed and is in clinical trial for patients with osteoporosis [46]. Anti-sclerostin antibody had potent bone anabolic effects and was well tolerated by the patients. Preclinical studies demonstrated that anti-sclerostin antibody treatment of murine models of MM can increase bone formation and may affect tumor burden. Several agents used to treat MM also have effects on bone. Bortezomib has been reported to have bone anabolic effects in patients with MM whose MM responded to bortezomib therapy [47], and transient increases in alkaline phosphatase activity have been shown in patients receiving bortezomib therapy. In addition there have been anecdotal reports of bone healing in patients receiving bortezomib-based therapies [48]. Furthermore, in preclinical studies, immunomodulatory agents such as pomalidomide can block osteoclast

differentiation [47]. Finally, the role of parathyroid hormone as an anabolic agent for MM bone disease remains unclear. Several groups reported the presence of the PTH receptor 1 on MM cells and found that parathyroid hormone-related protein, which activates the PTH receptor, can increase MM cell growth [49]. Other investigators failed to demonstrate PTH receptors on MM cells and preclinical studies have shown an anabolic effect of PTH in SCID-hu mouse model of MM [50].

Radiation Therapy and Surgery for Myeloma Bone Disease

Radiation therapy is frequently used to treat painful bone lesions in myeloma. However, it must be used sparingly because of its myelosuppressive effects. Thus, radiation therapy is used primarily to treat painful lesions, impending fracture and spinal cord compression [20]. Similarly, vertebroplasty and kyphoplasty are used to treat painful vertebral fractures in patients with myeloma [51]. In a randomized study for painful vertebral fractures in patients with myeloma, kyphoplasty was shown to be superior to conservative management, so that kyphoplasty is currently recommended by the IMWG guidelines.

Thromboprophylaxis for Myeloma Patients

Patients with MM have an increased risk of a thrombotic event during the course of their disease. In the original clinical trial of thalidomide for treatment of patients with MM, a 30% incidence of DVT was reported in patients receiving thalidomide [52]. Subsequent studies using combination chemotherapy regimens containing thalidomide also found a high rate of deep venous thrombosis (DVT) in these patients. Lenalidomide and pomalidomide are also associated with increased risk of DVT [52]. These results led to the development of thromboprophylaxis guidelines for patients receiving immunomodulatory agents [6]. These guidelines suggest that patients should receive thromboprophylaxis for at least the first four cycles of immunomodulatory agent-based therapy because the risk of DVT or pulmonary embolism (PE) is greatly increased during that time. The type of DVT prophylaxis is based on individual risk factors (e.g., age, obesity immobilization, history of DVT). Patients who have no or only one risk factor should receive prophylaxis with low-dose or standard-dose aspirin (81–325 mg per day) and patients with two or more risk factors should receive low-molecular-weight heparin or the equivalent or full-dose warfarin therapy to maintain the INR at 2–3. A recent abstract at the American society of clinical oncology found that the risk of VTE persistently changes throughout the disease process [53]. Lipe and coworkers found that VTE can occur in MM patients at a median time from diagnosis of 952 days, much longer than the IMWG guidelines recommend for thromboprophylaxis. Importantly, Lipe et al. found that of 60% of patients at high risk for thrombosis, only 16% ever received the recommended prophylactic therapy. These results suggest that VTE may occur later in MM than was previously thought, and that guidelines for thromboprophylaxis in MM may need to be adjusted. Finally, the use of erythropoiesis-stimulating agents (ESAs) also increases the risk of thrombosis in patients with MM. Katodritou and coworkers reported that ESAs reduced survival in MM patients compared to patients not receiving ESAs (and increased the risk of thrombosis in patients with MM) [54]. Thus, ESAs should be used with caution in patients with MM.

Neuropathy in Myeloma

Neurological complications in MM range from peripheral neuropathy to compression of the nerve roots or spinal cord. Compression of the nerve roots and spinal cord is an oncological emergency and can result from compression by vertebral and extramedullary plasmacytomas, respectively. Early recognition and treatment of spinal cord or nerve root compression are more important than the type of treatment in improving outcomes.

Radiculopathy

Compression of the nerve root by vertebral plasmacytomas, neuroforaminal stenosis, or vertebral fracture usually presents with back pain and a unilateral radiculopathy that may progress to sensory loss and weakness. At least 5% of MM patients suffer from spinal cord or cauda equina compression, and MM is responsible for 15% of hospitalization for malignant spinal cord compression [55]. Spinal cord compression often presents with progressively worsening back pain and may have a symmetric radiculopathy. The thoracic cord is most commonly affected and is associated with a sensory-level deficit. Bowel and bladder dysfunction is a late complication of spinal cord compression and is often preceded by symmetric pain and weakness. Unilateral complaints are rare but may be seen with lateralized cord compression. Complete spinal MRI with contrast is the preferred method of evaluating spinal cord compression, as CT does not clearly demonstrate the spinal cord or epidural space. Immediate treatment with corticosteroids, followed by radiation therapy, is the mainstay of treatment. MRI screening of MM patients for cord compression has not been found to be beneficial for detecting spinal cord compression [56].

Clinically detectable peripheral neuropathy is uncommon in MM at diagnosis and presents as a mild and slowly progressive symmetric neuropathy. Occurrence rates vary from 2% [5] to approximately 50% [57] of patients. Peripheral neuropathy in MM is often caused by accompanying amyloidosis or chemotherapy, and is a major presenting feature of POEMS syndrome. Cranial nerve involvement is rare and is usually a sign of progressive disease. Chemotherapy-induced peripheral neuropathy is a serious and potentially reversible side effect of treatment. The dose and duration of treatment are commonly affected by the neurotoxic profiles of these medications, which in severe cases may result in discontinuation of treatment. The clinical history and neurological examination are crucial for distinguishing CIPN from neuropathic involvement from direct nerve compression by MM. Neuropathy is a frequent complication of thalidomide- and bortezomib-based therapies.

Thalidomide is a potent antiangiogenic therapy that has been used for MM since 1998. Severe peripheral neuropathy occurs in approximately 30% of patients who were treated with doses of thalidomide greater than 200 mg/day [58]. Thalidomide-induced peripheral neuropathy presents as a symmetric distal loss of light touch and temperature that is painful. The neurotoxic effects of thalidomide are cumulative and dose dependent. In approximately 60% of patients, neuropathy results in lowering the dose or discontinuation of treatment [59]. Reduced starting doses of thalidomide of 100 mg/day for patients greater than 75 years old, and 200 mg/day for patients less than 75 years old, have reduced the incidence of severe peripheral neuropathy to less than 10%. However, there has been an overall increase in the incidence of mild-to-moderate peripheral neuropathy at these thalidomide doses. Although peripheral neuropathy is partially reversible with discontinuation of thalidomide, the long-term reversibility of neuropathy with discontinuation of thalidomide requires further long-term studies [60, 61]. The pathogenesis of thalidomide-induced peripheral neuropathy is poorly understood but antiangiogenic and inflammatory insults to the dorsal root ganglia may be the cause. This may explain the reduced sensory nerve action potentials with relative sparing of compound motor action potentials found in thalidomide-treated patients [62]. Other neurotoxic side effects of thalidomide include tremor (in up to 30%) and somnolence.

Bortezomib-associated peripheral neuropathy was recognized as an early side effect in the initial phase I trials. The incidence of bortezomib-induced peripheral neuropathy (BIPN) in phase II and phase III trials was 30–64%, and occurred in up to 46% of patients treated for newly diagnosed MM [62, 63]. BIPN is a subacute predominately sensory neuropathy of the feet and hands that is often painful. Small unmyelinated fibers are affected, causing prominent neuropathic pain in 25–80% of cases [64]. BIPN occurs in 21% and 37% of patients at the onset of treatment at doses of 1.0 mg/m^2, and 1.3 mg/m^2, respectively. The severity typically plateaus by the fifth cycle, with an approximate cumulative dose of 30 mg/m^2 [65]. The pathogenesis of BIPN remains unclear. The involvement of satellite and Schwann cells in BIPN in animal models supports the involvement of the dorsal root ganglia in the pathogenesis of the pain. Although the autonomic nervous system is also innervated by unmyelinated fibers, it is very rarely affected. The clinical presentation of a symmetric distal peripheral neuropathy with a temporal correlation to recent bortezomib treatment is suggestive of BIPN. Electrodiagnostic studies can provide objective evidence to aid in the diagnosis. However, the diagnosis can usually be made clinically. The neurologic examination is very important for distinguishing the cause of peripheral neuropathy. If signs of motor weakness are present, nerve root compression is more likely, since BIPN is primarily a sensory neuropathy. In patients with BIPN, nerve conduction studies show reduced sensory and motor action potentials, with mild slowing of the distal sensory and motor nerve velocities with increased distal motor latencies.

Bortezomib neurotoxicity is a potentially reversible complication that must be recognized early. Currently, there is no accepted proven treatment for BIPN. The mainstay of treatment remains bortezomib dose modification or discontinuation and neuropathic pain medication. Subcutaneous administration of bortezomib has dramatically reduced the incidence of neuropathy, and newer proteasome antagonists (carfilzomib and ixazomib) are less neurotoxic and better tolerated. Severe peripheral neuropathy was reported in less than 1% of patients in phase II trials of carfilzomib who were treated previously with bortezomib [66, 67]. There is a lack of evidence to support prevention of BIPN with neuroprotective agents [65].

Neurologic involvement is a hallmark of POEMS syndrome, a rare osteoscerotic form of MM, and is a major criterion for diagnosis. Approximately 50% of patients present with a distal symmetric neuropathy that ascends proximally. Eventually the vast majority of POEMS patients suffer from prominent sensorimotor neuropathy. Severe neuropathy has been reported in up to 76% of patients [68, 69]. Ocular involvement is common in approximately 2/3 of patients. Papilledema occurs in approximately half of those patients, and patients with papilledema have a worse prognosis [70]. There is also an increased risk of thromboembolic disease and cerebral infarction in POEMS patients. In a retrospective cohort study of 208 POEMS patients 19 (9%) developed cerebral infarction at a relatively young age [71]. The risk of cerebral infarction increased with the degree of thrombocytosis, and of plasma cell proliferation in the BM was also associated with increased stroke risk [71]. Electrodiagnostic studies are important to distinguish neuropathy due to POEMS from CIDP; in contrast to CIDP, prominent axonal

loss in the lower limbs rather than slow conduction of the distal nerve segments with conduction block occurs with POEMS. Polyneuropathy is commonly the initial presenting symptom of POEMS with up to 60% of patients with POEMS initially misdiagnosed with CIDP. Thus, distinguishing between CIDP and POEMS is critically important for treatment and prognosis. Nerve conduction studies in POEMS show predominate slowing of the nerve trunk when compared to distal nerve involvement.

Renal Dysfunction in Multiple Myeloma

The characteristic M paraprotein in MM, specifically the light-chain component, is nephrotoxic to the kidney, and frequently the kidney is the major target organ in MM. Up to 50% of patients have evidence of renal impairment at the time of diagnosis and about 10–15% of patients require renal replacement therapy [72]. Multiple studies found that renal impairment alone is a strong negative predictor of overall survival (OS) [4, 73]. In one study, the difference in median OS between patients with and without renal impairment was as high as 8.6 vs. 34.5 months ($P < 0.001$) The same study also showed markedly improved OS if renal impairment was reversible [4]. The Nordic MM study group also found that severity of renal impairment was a determinant of OS MM [73]. Hypercalcemia, lower proteinuria (<1 g/day), and low serum creatinine were positive prognostic factors. Thus, early detection of MM-induced kidney disease is extremely important.

The IMWG defines renal injury as either an elevation of serum creatinine >2 mg/dL or a reduced creatinine clearance (CrCl) of <40 mL/min/1.73m^2, due to MM [74]. CrCl should be assessed using the Modification of Diet in Renal Disease (MDRD) formula or the Chronic Kidney Disease Epidemiology Collaboration (CKD-EPI) equation as these most accurately predict the clearance obtained from inulin-based glomerular filtration rate (GFR) estimation [75, 76]. The CKD-EPI group further suggests that an equation based on creatinine and cystatin C, which also reflects tumor burden, is more accurate, but this remains to be validated in larger studies [75]. The use of eGFR should be restricted to stable patients and not for those presenting with an acute kidney injury (AKI). RIFLE (Risk, Injury, Failure, Loss and End-Stage Kidney Disease) criteria and Acute Kidney Injury Network classification should be used for assessing the severity of AKI [77].

Initial Diagnostic Workup of Renal Injury in Myeloma

All patients with symptomatic MM should have an initial laboratory panel that includes serum creatinine, GFR estimation, electrolytes, serum free light chains (SFLC), and 24-h urine collection for proteinuria and urine electrophoresis. The presence of selective proteinuria composed almost entirely of light chains can be attributed to myeloma cast nephropathy (MCN), whereas the presence of significant albuminuria suggests alternative diagnoses that require further investigation [78]. A renal biopsy should be considered to exclude monoclonal immunoglobulin deposition disease, amyloidosis, or other underlying causes of renal impairment in the presence of nonselective proteinuria [78–80]. If amyloidosis is strongly suspected, a fat pad aspirate should be done prior to kidney biopsy, since a diagnosis of amyloidosis can be obtained in about 70% of cases with the less invasive procedure [80].

Pathogenesis of Myeloma Kidney Disease

Free light chains circulating as monomers (predominantly K, 25KD) or dimers (predominantly λ, 50KD) are freely filtered by the glomerulus. In the proximal tubule cells (PTC) they are reabsorbed by receptor-mediated endocytosis [81]. This glycoprotein receptor cubilin-mediated endocytosis incites a proinflammatory response that induces release of IL-6, IL-8, and monocyte chemoattractant protein 1 (MCP-1) via activation of nuclear factor kappa light-chain enhancer of activated B-cells (NF-kB) in the PTC [82]. Similar to other proteinuric diseases, excess light chains induce factors that promote interstitial injury and fibrosis, as well as direct DNA injury and induction of apoptosis [83].

Once the PTC is injured, LCs overflow into the distal lumen and interact with the Tamm-Horsfall protein (THP) secreted by the thick ascending limb (TAL). This leads to formation of the MM cast that is responsible for the presentation of MCN. The variability in cast formation by this interaction can be explained by the variation in the complementarity-determining region 3 (CDR3) of different LCs [84]. While urinary light chains are required for the formation of casts, the type or quantity of LC does not correlate with the severity of cast formation. Urinary LC quantity, however, is predictive of response to therapy and risk for renal failure. Tubular solute composition and flow rates are other factors that also influence cast formation. Obstructed tubules in turn induce an intense inflammatory response probably related to urine leak into the interstitium [85].

Common Patterns of Renal Insufficiency in Myeloma

Myeloma cast nephropathy (MCN) is the most common histologic finding with autopsy studies reporting rates between 30 and 50% of all MM patients with renal failure [86–88]. MCN almost always occurs in the presence of high free LC

burden and typically when serum FLC levels are >100 mg/dL [74, 89]. Almost all MM patients with significant renal impairment will also have high urine FLC levels [90]. The serum and urine FLC levels can be prognostic indicators in MCN [91, 92]. The hallmark of MCN is tubular obstruction due to formation of LC casts, as described previously, from the interaction of Tamm-Horsfall protein (THP) and monoclonal LCs. Factors that can influence formation of casts include urinary concentration of THP, sodium, calcium, pH, urine flow rate, and diuretic use [85].

MCN typically presents with acute renal failure; however, oliguric AKI is a feature in about half of the cases even with very high levels of serum creatinine [93]. The progression of AKI is rapid [94], with volume depletion and hypercalcemia being the most common precipitating factors. Other common risk factors for AKI MM include use of IV contrast media, presence of infection, and nonsteroidal anti-inflammatory drugs (NSAIDs) used for bone pain. One of the initial indicators for MCN is the presence of heavy proteinuria that is disproportionate to the albuminuria detected on a urine dipstick. The median amount of proteinuria is about 2 g/24 h and is almost exclusively Bence Jones protein with albuminuria contributing <10%.

Early identification of MCN is essential for the treatment of MCN since delay in decreasing light-chain burden is associated with lower kidney recovery rates [92]. Treatment is focused on elimination of the precipitating agent and rapid reduction of the paraprotein. This can be achieved with chemotherapy or extracorporeal removal via therapeutic plasma exchange (TPE). While renal replacement therapy (RRT) may be required in certain cases, the indication for initiation of RRT is similar to other AKI states. Role of TPE in the management of MCN and reduction of FLC burden remains controversial. Three randomized controlled trials have published conflicting results [95–97]. The largest trial showed no benefit but was limited by the absence of histologic diagnosis of MCN. A meta-analysis of the three trials showed higher rates of dialysis independence at 6 months for patients when TPE was combined with chemotherapy but no change in overall survival with use of TPE [98]. Arguments against using TPE include the absence of FLC monitoring in previous randomized trials and introduction of newer chemotherapeutic agents with high response rates that did not significantly improve with addition of TPE [99, 100].

The role of high-cutoff hemodialysis (HCO-HD) in treatment of patients with renal insufficiency is being evaluated in two large randomized controlled trials: EuLITE study (European Trial of Free Light Chain Removal by Extended Hemodialysis in Cast Nephropathy; NCT00700531) and the French MYRE study (Studies in Patients with MM and Renal Failure Due To MM Cast Nephropathy; NCT01208818). Both these trials include patients on a bortezomib-based regimen. A prior study examining the use of HCO-HD in combination with chemotherapy in 67 MM patients showed encouraging results, with a sustained reduction in FLCs by day 12 in 67% of patients and dialysis independency in 63% [101]. However, sustained reduction in FLC requires effective chemotherapy. The choice of regimen is oftentimes guided by the presence of renal failure and nonrenally cleared drugs like bortezomib and thalidomide are preferred.

General Principles of Treatment Renal Insufficiency in Myeloma

Initial treatment should include administration of intravenous fluids to achieve high urine, reduction of calcium levels in patients with hypercalcemia, treatment of infection, and avoidance of nephrotoxic agents. However, there is no established role for urine alkalization in treatment of RI [102]. Bisphosphonates can be used for hypercalcemia; however, pamidronate and zoledronic acid should be avoided in patients with CrCl <30 mL/min. Pamidronate has been associated with development of collapsing variant of focal and segmental glomerulosclerosis [103], whereas zoledronic acid is associated with development of ATN [104]. Denosumab may be helpful in this subgroup with close monitoring of serum calcium levels. The role of mechanical treatments, which include therapeutic plasma exchange (TPE) or high-cutoff hemodialysis (HCO-HD), in treatment of MM is limited to MM cast nephropathy (MCN) (see above).

Systemic chemotherapy should be started immediately to reduce FLC burden and provides the best chance of renal recovery, with bortezomib in combination with dexamethasone usually employed [72]. Bortezomib-based therapy has significantly higher rates of renal recovery (≥partial renal response) compared to thalidomide- or lenalidomide-based regimens (77% vs. 55% vs. 43%, respectively) in a large retrospective analysis of 133 patients [105]. Carfilzomib, a second-generation proteasome inhibitor, has shown good safety and efficacy when used in patients with RI, and progression-free survival was better with carfilzomib when compared to bortezomib [106]. High-dose corticosteroids (equivalent to dexamethasone 160 mg or greater over 4 days) can be effective for rapid recovery of renal function regardless of the initial chemotherapy regimen [105]. High-dose steroids should be used in the initial month of therapy for MM. Thalidomide can be used in renal insufficiency without dose adjustment; however there have been reports of hyperkalemia in patients receiving dialysis [107, 108]. The renal recovery rates with use of thalidomide have been as high as 75% in newly diagnosed MM and up to 60% in patients with relapse/refractory disease [105, 107, 109].

Lenalidomide requires dose adjustment in renal insufficiency and response to treatment is not as robust. Autologous stem cell transplant (ASCT) can be used in MM even in presence of severe RI requiring dialysis but requires dose adjustment of the high-dose melphalan [110]. RI at the time of transplantation, however, is associated with higher risk of transplant-related mortality (4% vs. <1%) [110, 111].

AL Amyloidosis

Amyloidosis represents the most common glomerular lesion in MM. AL amyloidosis is a systemic disease with deposition of congophilic fibrils in soft tissue. The fibrils are composed of light chains (AL), heavy chains (AH), or intact immunoglobulins (ALH) [112]. AL amyloidosis is the most common subtype accounting for >95% of cases. Autopsy studies report rates of 5–15% for AL amyloidosis [86–88]. MM is diagnosed in <10% of cases of AL amyloidosis. About 50% of patients present with renal failure. Proteinuria is present in >70% of the cases while about a fourth of the cases present with nephrotic syndrome [113]. As opposed to MCN, the proteinuria is predominantly albumin [78]. Rarely, amyloidosis will present with renal dysfunction in the absence of proteinuria as in vascular limited amyloidosis or diabetes insipidus. Kidney involvement can at times be the only presentation of amyloidosis. A fat pad biopsy can provide a diagnosis in about 70% of cases, whereas the remaining will require kidney biopsy [78]. Kidney response in amyloidosis is predictive of OS [114, 115]. Kidney transplantation can be considered in combination with chemotherapy and ASCT as one of the therapeutic options in patients with AL amyloidosis [116].

Kidney Transplantation in Myeloma

Kidney transplantation is challenging in patients with MM since the transplantation and ensuing use of immunosuppressive medications are thought to portend a high risk for disease recurrence and infections. Most centers require a treatment-free remission period of 3–5 years. The initial lesion can be predictive of graft survival and recurrence. Patients with MCN have a lower risk of graft recurrence if MM stays in remission [117]. In patients with AL amyloidosis, specifically those without significant cardiac involvement, kidney transplantation in combination with chemotherapy and ASCT has shown favorable outcomes [116]. Renal transplantation in patients with MIDD is not recommended unless complete response is noted, as recurrence rates are about 80% in patients who do not have a complete response to treatment [118].

Conclusion

Thus, supportive care for MM patients becomes increasingly important, if MM patients are to enjoy a higher quality of life. MM patients are living longer, receiving more intensive therapy, and are potentially curable.

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32 POEMS Syndrome

Angela Dispenzieri

Introduction

POEMS syndrome is a rare paraneoplastic syndrome due to an underlying plasma cell disorder. The acronym, which was coined by Bardwick in 1980 [1], refers to several, but not all, of the features of the syndrome: polyradiculoneuropathy, organomegaly, endocrinopathy, monoclonal plasma cell disorder, and skin changes. Other important features not included in the POEMS acronym include papilledema, extravascular volume overload, sclerotic bone lesions, thrombocytosis/ erythrocytosis (P.E.S.T.), elevated vascular endothelial growth factor (VEGF) levels, a predisposition towards thrombosis, and abnormal pulmonary function tests. There is a Castleman disease variant of POEMS syndrome that may be associated with a clonal plasma cell disorder. Other names of the POEMS syndrome that are less frequently used are osteosclerotic myeloma, Takatsuki syndrome, Crow-Fukase syndrome, and PEP syndrome [2, 3]. A national survey conducted in Japan in 2003 showed a prevalence of approximately 0.3 per 100,000 [4].

The pathogenesis of the syndrome is not understood. Distinctive presenting characteristics of the syndrome that differentiate POEMS syndrome from standard multiple myeloma (MM) include the following: (1) dominant symptoms have little to nothing to do with bone pain, extremes of bone marrow infiltration by plasma cells, or renal failure; (2) dominant symptoms are typically neuropathy, endocrine dysfunction, and volume overload; (3) VEGF levels are high; (4) sclerotic bone lesions are present in the majority of cases; (5) overall survival is typically superior; and (6) lambda clones predominate [5]. VEGF is the cytokine that correlates best with disease activity, although it is likely not the driving force of the disease based on the mixed results seen with anti-VEGF therapy [6]. Little is known about the plasma cells in POEMS syndrome except that more than 95% of the time they are lambda light chain restricted with restricted immunoglobulin light-chain variable gene usage (*IGLV1*) [7, 8].

Diagnosis of POEMS Syndrome

The diagnosis is made based on a composite of clinical and laboratory features. The most notable symptoms include the constellation of neuropathy and any of the following: monoclonal protein (especially lambda light chain); thrombocytosis; anasarca; or papilledema. All the features of the acronym are not required to make the diagnosis (Table 32.1). A good history and physical examination followed by appropriate testing—most notably radiographic assessment of bones [9], measurement of VEGF [10–14], and careful analysis of a bone marrow biopsy [15]—can differentiate this syndrome from other conditions like chronic inflammatory polyradiculoneuropathy (CIDP), monoclonal gammopathy of undetermined significance (MGUS) neuropathy, and immunoglobulin light-chain amyloid neuropathy. Figure 32.1 demonstrates several classic findings among patients with POEMS syndrome.

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Table 32.1 Criteria for the diagnosis of POEMS syndrome[a]

		% Affected[b]
Mandatory major criteria (both required)	1. Polyradiculoneuropathy (typically demyelinating)	100
	2. Monoclonal plasma cell disorder (almost always λ)	100[c]
Other major criteria (one required)	3. Castleman disease[d]	11–25
	4. Sclerotic bone lesions	27–97
	5. Vascular endothelial growth factor elevation[e]	
Minor criteria (one required)	6. Organomegaly (splenomegaly, hepatomegaly, or lymphadenopathy)	45–85
	7. Extravascular volume overload (edema, pleural effusion, or ascites)	29–87
	8. Endocrinopathy (adrenal, thyroid,[f] pituitary, gonadal, parathyroid, pancreatic[f])	67–84
	9. Skin changes (hyperpigmentation, hypertrichosis, glomeruloid hemangiomata, plethora, acrocyanosis, flushing, white nails)	68–89
	10. Papilledema	29–64
	11. Thrombocytosis/polycythemia[g]	54–88
Other symptoms and signs	Clubbing, weight loss, hyperhidrosis, pulmonary hypertension/restrictive lung disease, thrombotic diatheses, diarrhea, low vitamin B_{12} values	

POEMS polyneuropathy, organomegaly, endocrinopathy, M-protein, skin changes
[a]The diagnosis of POEMS syndrome is confirmed when both of the mandatory major criteria, one of the three other major criteria, and one of the six minor criteria are present
[b]Summary of frequencies of POEMS syndrome features based on largest retrospective series [2, 3, 16–18, 27]
[c]Takasuki and Nakanishi series are included even though only 75% of patients had a documented plasma cell disorder.
[d]There is a Castleman disease variant of POEMS syndrome that occurs *without* evidence of a clonal plasma cell disorder that is not accounted for in this table. This entity should be considered separately
[e]A plasma VEGF level of 200 pg/mL is 95% specific and 68% sensitive for a POEMS syndrome [14]
[f]Because of the high prevalence of diabetes mellitus and thyroid abnormalities, this diagnosis alone is not sufficient to meet this minor criterion
[g]Approximately 50% of patients will have bone marrow changes that distinguish it from a typical MGUS or myeloma bone marrow
Reproduced with permission from Dispenzieri, A., How I treat POEMS syndrome. Blood, 2012. ***119****(24): p. 5650–8*

Clinical and Laboratory Presentation

The peripheral neuropathy is the dominant characteristic [2, 3, 16–18], and it is ascending, symmetrical, and affecting both sensation and motor function [19]. In our experience, pain may be a dominant feature in about 10–15% of patients, and in one report as many as 76% of patients had painful neuropathy [4, 20]. Nerve conduction studies in patients with POEMS syndrome show slowing of nerve conduction that is more predominant in the intermediate than distal nerve segments as compared to CIDP, and there is more severe attenuation of compound muscle action potentials in the lower than upper limbs [4, 21, 22]. In contrast to CIDP, conduction block is rare [4, 22]. The conduction findings suggest that demyelination is predominant in the nerve trunk rather than the distal nerve terminals, and axonal loss is predominant in the lower limb nerves [4]. Axonal loss is greater in POEMS syndrome than it is in CIDP [22]. The nerve biopsy is not specific, but uncompacted myelin lamellae, endothelial cytoplasmic enlargement, opening of the tight junctions between endothelial cells and presence of many pinocytic vesicles adjacent to the cell membranes, and absence of macrophage-associated demyelination have been described [23, 24]. As compared to CIDP, POEMS syndrome demonstrates more axonal degeneration and epineurial neovascularization but less endoneurial inflammation and onion-bulb formation [25].

Depending on the series, 45–85% of patients will have any combination of splenomegaly, hepatomegaly, and/or lymphadenopathy. Lymph nodes may appear reactive or reveal frank Castleman disease or merely “Castleman’s disease-like” changes. Between 11 and 30% of POEMS patients with documented clonal plasma cell disorder also have documented Castleman disease or Castleman-like histology [6]. Among individuals with POEMS who undergo lymph node biopsy, about 50% show angiofollicular hyperplasia typical of Castleman disease [3, 18], and 84% are of these are hyaline vascular type [18]. Only those with peripheral neuropathy AND a plasma cell clone should be classified as standard POEMS syndrome; without both, patients can be classified as Castleman disease variant of POEMS if they have other POEMS features [26].

Endocrinopathy is a central but poorly understood feature of POEMS. In one series [27], approximately 84% of patients had a recognized endocrinopathy, with hypogonadism as the most common endocrine abnormality, followed by thyroid

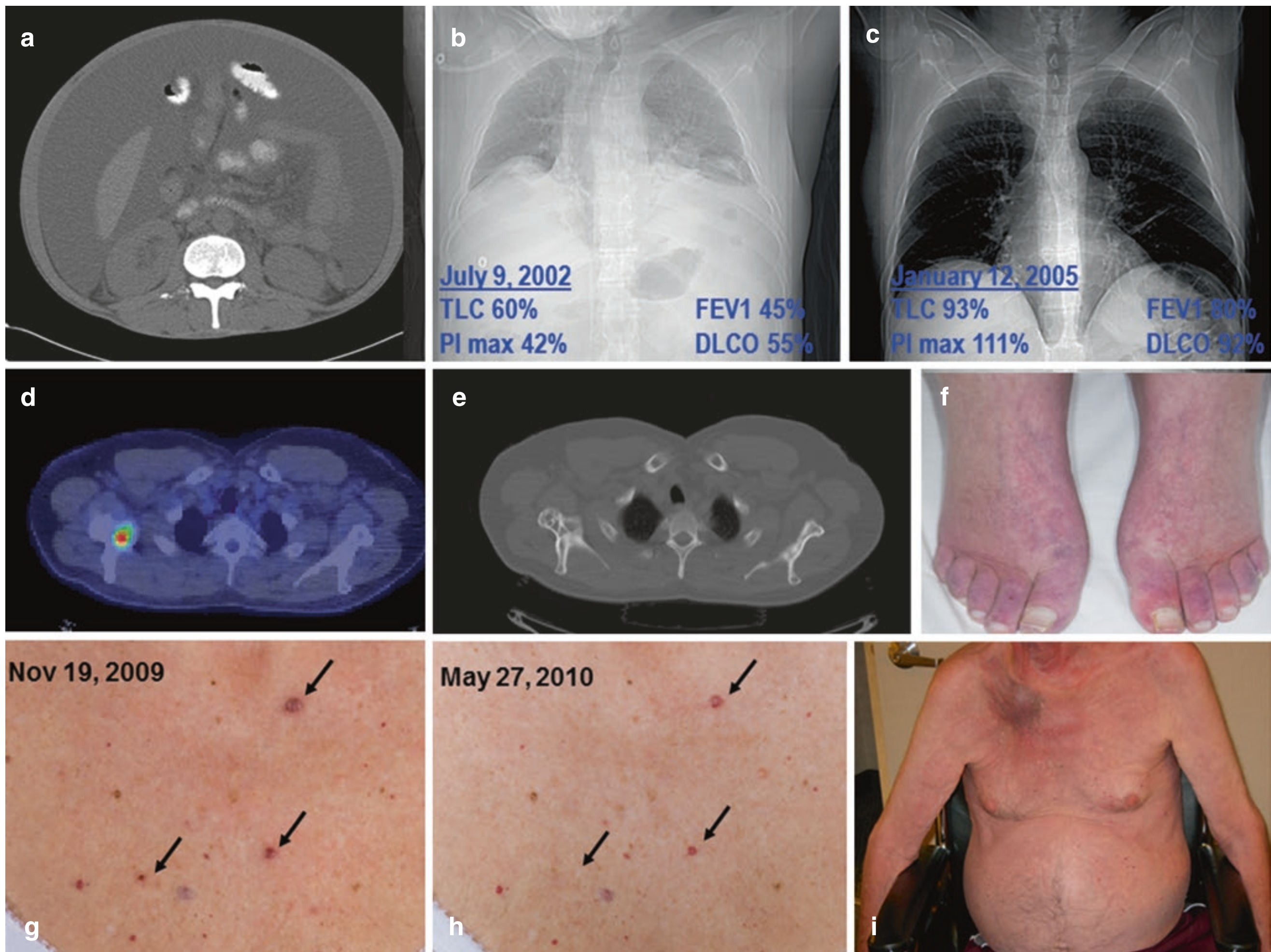

Fig. 32.1 Classic findings of POEMS syndromes. *Reproduced with permission from Dispenzieri, A., How I treat POEMS syndrome. Blood, 2012.* ***119****(24): p. 5650–8.* (**a**) Massive ascites and lipodystrophy. (**b**) Chest radiograph and pulmonary function test results demonstrating reduced lung volumes due to neuromuscular weakness, small effusions, and reduced diffusing capacity of carbon monoxide. (**c**) Improved chest radiograph and pulmonary function tests 2.5 years after ASCT (same patient as **h**). (**d**) Fusion CT PET of mixed lytic/sclerotic lesion in right scapula. (**e**) Bone windows of CT of mixed lytic/sclerotic lesion in right scapula. (**f**) Hyperemia of extremities and white nails. (**g**) Outcropping of cherry angiomata at diagnosis. (**h**) Shrinkage and disappearance of cherry angiomata after radiation to solitary osteosclerotic lesion right femur. (**i**) Plasmacytoma right scapula with overlying erythema as well as gynecomastia, muscle wasting, and ascites. Also present but unrelated is florid tinea corporis due to chronic steroid used for the incorrect diagnosis of CIDP

abnormalities, glucose metabolism abnormalities, and lastly adrenal insufficiency. The majority of patients have evidence of multiple endocrinopathies in the four major endocrine axes (gonadal, thyroid, glucose, and adrenal). Gynecomastia may be present on physical examination.

The characteristic skin changes include hyperpigmentation, a recent outcropping of hemangioma, hypertrichosis, dependent rubor and acrocyanosis, white nails, sclerodermoid changes, facial lipodystrophy, flushing, or clubbing [6]. Rarely calciphylaxis is also seen.

Papilledema is present in at least one-third of patients [28]; the majority of these patients do not have specific symptoms relating to this finding but a minority will report blurred vision, diplopia, or ocular pain. Peripheral edema, ascites, and effusions are the symptoms and signs that cause the next most morbidity after the peripheral neuropathy. The manifestations of extravascular overload occur in 29–87% of patients with POEMS syndrome and are not typically associated with severe hypoalbuminemia. Severe third spacing can lead to worsening renal function. Serum creatinine levels are normal in most cases, but serum cystatin C, which is a surrogate marker for renal function, is high in 71% of patients [29]. In a series from China, 22% of patients had a creatinine clearance (CrCl) of less than 60 mL/min including 8% with a CrCl of less than 30 mL/min; 10% had microhematuria [30]. The renal histologic findings are diverse with membranoproliferative features and evidence of endothelial injury being most common [31].

Finding the monoclonal plasma cell disorder can sometimes be a challenge. In one series, only 53% of patients had a positive protein electrophoresis, with another 31% being immunoelectrophoresis positively only, and 16% had their clone discovered only by either bone marrow biopsy or biopsy of a plasmacytoma, i.e., bone lesion [17]. The CBC is notable for an absence of cytopenias. Nearly half of patients will have thrombocytosis or erythrocytosis [17]. In a series from China, 26% of patients had anemia, which the authors attributed to impaired renal function [18]. Their series was enriched with Castleman disease cases (25%), which may have also contributed to this unprecedentedly high rate of anemia.

The bone marrow biopsy reveals megakaryocyte hyperplasia and megakaryocyte clustering in 54% and 93% of cases, respectively [15]. These megakaryocyte findings are reminiscent of a myeloproliferative disorder, but *JAK2* V617F mutation is uniformly absent. One-third of patients do not have clonal plasma cells on their iliac crest biopsy. These are the patients who present with a solitary or "multiple solitary plasmacytomas." The median percent of plasma cells observed is less than 5%. Immunohistochemical staining is more sensitive than is six-color flow since the former provides information on bone marrow architecture, which is key in making the diagnosis in nearly half of cases. In our study of 67 pretreatment bone marrow biopsies from patients with POEMS syndrome, lymphoid aggregates were found in 49% of cases. Of these, there was plasma cell rimming in all but one; and of these 32 cases, 31 were clonal lambda and 1 was kappa. This finding was not seen in bone marrows from normal controls or from patients with MGUS, multiple myeloma, or amyloidosis. Overall, only 8/67 (12%) of POEMS cases had normal iliac crest bone marrow biopsies, i.e., no detectable clonal plasma cells, no plasma cell-rimmed lymphoid aggregates, and no megakaryocyte hyperplasia.

Osteosclerotic lesions occur in approximately 95% of patients, and can be confused with benign bone islands, aneurysmal bone cysts, non-ossifying fibromas, and fibrous dysplasia [3, 17]. Some lesions are densely sclerotic, while others are lytic with a sclerotic rim, while still others have a mixed soap-bubble appearance. Occasionally patients have a lytic lesion without any evident sclerosis. Bone windows of CT body images are often very informative [32], often even more so than FDG uptake, which can be variable and most useful when there is an obvious lytic component to the bone lesion.

Plasma and serum levels of VEGF are markedly elevated in patients with POEMS [14, 33, 34] and correlate with the activity of the disease [10, 11, 14, 34]. The principal isoform of VEGF expressed is VEGF165 [10]. VEGF levels are independent of M-protein size [10]. IL-1β, TNF-α, IL-6, and IL-12 levels are often also increased [35]. Serum VEGF levels are 10–50 times higher plasma levels of VEGF [36]. Our group has demonstrated that a plasma VEGF level of 200 pg/mL had a specificity of 95% with a sensitivity of 68% in support of a diagnosis of POEMS syndrome.

Respiratory complaints are usually limited given patients' neurologic status impairing their ability to induce cardiovascular challenges [37]. The pulmonary manifestations are protean, including pulmonary hypertension, restrictive lung disease, impaired neuromuscular respiratory function, and impaired diffusion capacity of carbon monoxide, but improve with effective therapy [18, 37, 38]. Pulmonary hypertension has been reported to occur in 27% of unselected patients with POEMS syndrome [39]. Nail clubbing is seen in about 4–49% of cases [3, 37].

Patients are at increased risk for arterial and/or venous thromboses during their course, with nearly 20% of patients experiencing one of these complications [5, 40]. Ten percent of patients present with a cerebrovascular event, most commonly embolic or vessel dissection and stenosis [41]. Thrombocytosis and increased bone marrow infiltration are associated with risk for cerebrovascular accidents [41]. Aberrations in the coagulation cascade have been implicated in POEMS syndrome, but are not usually clinically apparent [24].

Treatment of Poems Syndrome

Despite the relationship between disease response and dropping levels of VEGF, the most experience with successful outcomes has been associated with directing therapy at the underlying clonal plasma cell disorder rather than solely targeting VEGF with anti-VEGF antibodies. The treatment algorithm is based on the extent of the plasma cell infiltration (Fig. 32.2). The approach to therapy differs based on whether there is bone marrow involvement as determined by blind iliac crest sampling [42].

The course of POEMS syndrome is usually chronic with an estimated 10-year survivorship rate of 77–79% [43, 44] and of more than 90% for those who undergo ASCT [17, 37, 44]. The number of POEMS features does not affect survival [5, 16]. Baseline risk factors for inferior survival have included fingernail clubbing, extravascular volume overload—i.e., effusions, edema, and ascites [17]—low serum albumin [44], coexistent Castleman disease [18], and respiratory symptoms [37]. In a recent publication from China of 362 patients, factors associated with inferior survival were age (40% of patients HR 4.1 [95%CI 1.4, 11.8]), pulmonary hypertension (20%, HR 4.0 [95%CI 1.4, 11,0]), pleural effusion (40%; HR 3.8 [95%CI 1.2, 11.8]), and eGFR <30 mL/min/1.73 m^2 (6%; HR 8.2 [95%CI 2.2, 31.2]).

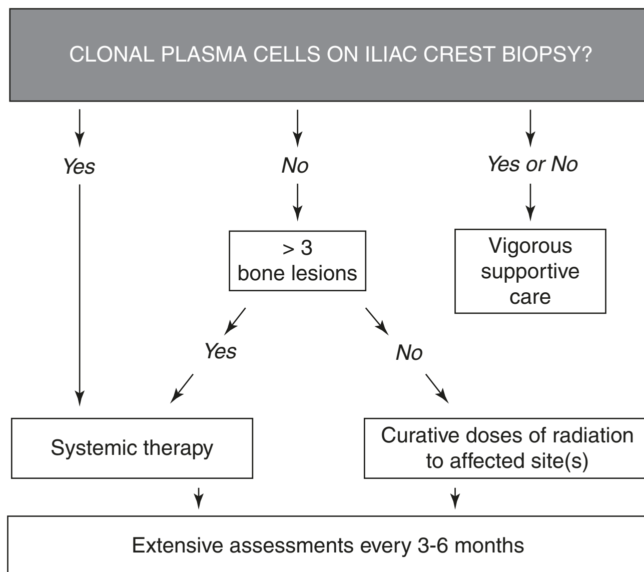

Fig. 32.2 Algorithm for the treatment of POEMS syndrome. *Reproduced with permission from Dispenzieri, A., How I treat POEMS syndrome. Blood, 2012.* ***119****(24): p. 5650–8*

Management of POEMS Syndrome Without Disseminated Bone Marrow Involvement

In the case of patients with an isolated bone lesion without clonal plasma cells found on iliac crest biopsy, radiation is the recommended therapy as it is in the case of a more straightforward solitary plasmacytoma of bone. Not only does radiation to an isolated (or even two or three isolated) lesion(s) improve the symptoms of POEMS syndrome over the course of 3–36 months, but it can also be curative.

Management of POEMS Syndrome with Disseminated Bone Marrow Involvement

Typically, once there is disseminated disease identified, systemic therapy is recommended with the caveat that large bony lesions with a significant lytic component may require adjuvant radiation therapy [42]. Decisions about adjuvant radiation should be made on a case-by-case basis, and typically not until a minimal of 6 months after completing chemotherapy. There is a lag between completion of successful therapy and neurologic response, often with no discernible improvement until 6 months after completion of therapy. Maximal response is not seen until 2–3 years hence. Other features like anasarca, papilledema, and even skin changes typically improve sooner. Optimal FDG-PET response may also lag by 6–12 months.

Table 32.2 Activity of therapy for the treatment of POEMS syndrome

Regimen	Outcome
Radiation	50–70% of patients have significant clinical improvement
Melphalan-dexamethasone	81% hematologic response rate; 100% with some neurologic improvement
Corticosteroids	50% of patients have significant clinical improvement
Cyclophosphamide-dexamethasone	At least 50% of patients have significant improvement
ASCT	100% of surviving patients have significant clinical improvement
Thalidomide-dexamethasone	Reported responses in 12 patients, but not recommended as first line due to risk of neuropathy. Additional 25 patients treated in randomized trial with crossover revealed improved VEGF and motor function [49]
Lenalidomide-dexamethasone	Reported responses in majority of patients (more than 60 patients reported)
Bortezomib	Used as single agent ($n = 1$), with dexamethasone ($n = 2$), with cyclophosphamide and dexamethasone ($n = 1$), and with doxorubicin and dexamethasone ($n = 1$). Reported responses in all
Bevacizumab	Two out of three using it as single agent died within weeks; one improved. Two other patients using it as “salvage” improved, but relapsed and died despite continued therapy, normal VEGF at 3.5 and 5.5 years. Six other cases of use with or after other alkylator-based therapy yielded one death and four patients with improvement

Modified from Dispenzieri [6]

Since there is a paucity of clinical trials among patients with POEMS syndrome [6], treatment recommendations are largely based on case series and anecdote. The treatment armamentarium is borrowed from other plasma cell disorders. Table 32.2 summarizes regimens and observed outcomes. Corticosteroids may provide symptomatic improvement, but response duration is limited. The most experience has been with alkylator-based therapy, either low dose or high dose with peripheral blood stem cell transplant. The first prospective clinical trial to treat POEMS syndrome was reported from China [45]. Thirty-one patients were treated with 12 cycles of melphalan and dexamethasone and 81% of patients achieved hematologic response, 100% had VEGF response, and 100% had at least some improvement in neurologic status. At 21 months, 100% were progression free and alive. Personal experience and retrospective reports of the use of cyclophosphamide-based therapy is effective [42].

High-dose alkylator with peripheral blood stem cell transplant is also quite effective, but selection basis may confound these reports [6]. Case series suggest that 100% of patients achieve at least some neurologic improvement. Doses of melphalan ranging from 140 to 200 mg/m^2 have been used, with the lower doses used for sicker patients. Of the 59 patients with POEMS syndrome treated at the Mayo Clinic Rochester, progression-free survival was 98%, 94%, and 75% at 1, 2, and 5 years, respectively [46]. Symptomatic progressions were rare, whereas radiographic and VEGF progressions were most common. Treatment-related morbidity and mortality can be minimized by recognizing and treating an engraftment-type syndrome characterized by fevers, rash, diarrhea, weight gain, and respiratory symptoms and signs that occur anytime between days 7 and 15 post-stem cell infusion [47]. In a recent EBMT series, engraftment syndrome was reported in 23% of patients. Survival figures were comparable to that seen in the Mayo series [48].

There is one randomized trial for patients with POEMS syndrome. Misawa and colleagues conducted a randomized, double-blind, placebo-controlled phase 2/3 trial for patients with POEMS syndrome who were not candidates for ASCT [49]. Twenty-five patients were randomized to either daily thalidomide plus 4 days of dexamethasone every 4 weeks or placebo and 4 days of dexamethasone for 24 weeks; thereafter all patients received open-label, single-agent thalidomide for 48 weeks. Patients treated with thalidomide had higher rates of VEGF reduction, greater change in summated muscle test scores, and smaller changes in SF-36 QoL physical function and physical scores. Not surprisingly, side effects including sinus bradycardia, constipation, and mild sensory neuropathy were more frequent in patients treated with thalidomide.

Lenalidomide has also yielded favorable results, seemingly with fewer side effects. The French have reported in abstract form their results of a Phase 2 study of lenalidomide and dexamethasone for 2 cycles as neoadjuvant therapy preceding radiation or high-dose therapy or as primary therapy as 9 cycles followed by 12 cycles of single-agent lenalidomide [50]. They treated 27 patients: 10 pre-radiation therapy; 8 pre-ASCT; and 9 primary therapy. Although follow-up is short, the authors report that several patients had rapid neurological response, no patient had died, and one patient had progressed. These results are similar to case reports and case series [51–53]. In the largest case series of 20 patients [53], all patients responded, but 4 patients relapsed 3–10 months after the end of treatment. Another retrospective case series of 12 patients with relapsed or refractory POEMS syndrome treated with lenalidomide and dexamethasone reported a 2-year PFS and OS of 92% [54]. A systematic review of lenalidomide use in patients with POEMS has been published [55], which estimates a 1- and 2-year PFS of 92 and 42% using lenalidomide. Given the intrinsic risk patients with POEMS syndrome have for thrombosis, it is imperative that at least an aspirin be used for prophylaxis. The use of low-molecular-weight heparin or warfarin should be balanced against fall risk.

Like thalidomide and lenalidomide, bortezomib can have anti-VEGF and anti-TNF effects. Enthusiasm for thalidomide and bortezomib should be tempered by the high rate of peripheral neuropathy induced by these drugs. Bortezomib use has been reported in a handful of patients, with favorable results, especially those with severe ascites [6]. Although an anti-VEGF strategy is appealing, the results with bevacizumab have been mixed. Most of these reports include patients who had also received either radiation or alkylator during and/or predating the bevacizumab had benefit [42].

Both our experience and the literature would support that single-agent IV IG or plasmapheresis is not helpful. Other treatments like interferon-alpha, tamoxifen, trans-retinoic acid, ticlopidine, argatroban, and strontium-89 have been reported as having activity mostly as single-case reports.

Managing Symptoms of Disease

Attention to supportive care is imperative. Orthotics, physical therapy, and CPAP all play an important role in patients' recovery. Ankle foot orthotics can increase mobility and reduce falls. Physical therapy reduces the risk for permanent contractures and leads to improved function both in the long and short terms. For those with severe neuromuscular weakness, CPAP and/or biBAP provides better oxygenation and potentially reduces the risk complications associated with hypoventilation like pulmonary infection and pulmonary hypertension.

Monitoring Response

Patients must be followed carefully on a quarterly basis tracking the status of deficits comparing these to baseline [46]. VEGF responses may occur as soon as 3 months [56], but they can be delayed. VEGF is an imperfect marker since discordance between disease activity and response has been reported [57], so trends rather than absolute values should direct therapeutic decisions. Serum M-protein responses by protein electrophoresis, immunofixation electrophoresis, or serum immunoglobulin free light chains also pose a challenge. The size of the M-protein is typically small making standard multiple myeloma response criteria inapplicable in most cases. In addition, patients can derive very significant clinical benefit in the absence of an M-protein response [45, 47]. Finally, despite the fact that the immunoglobulin free light chains are elevated in 90% of POEMS patients, the ratio is normal in all but 18% [29], making the test of limited value for patients with POEMS syndrome.

Conclusion

POEMS syndrome is a rare disorder. There is much to be learned about the pathogenesis of the disease. From a practical standpoint, however, one of the biggest challenges is that diagnosis is often delayed, which results in increased morbidity. Hematologic, clinical, VEGF, and PET scan responses can be achieved with a multitude of therapies, but there are no randomized data to determine which therapies are best in terms of rapid, deepest, and most durable responses. If the diagnosis is made too late, patients may be left with some extent of irreversible nerve damage, so neurologists and hematologists should be vigilant of POEMS in their differential diagnosis of patients with lambda-restricted plasma cell disorder, peripheral neuropathy, and other POEMS syndrome features.

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Check for updates

Waldenstrom's Macroglobulinemia

33

Steven P. Treon, Giampaolo Merlini,
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Background

Waldenstrom's macroglobulinemia (WM) is a lymphoid neoplasm resulting from the accumulation, predominantly in the marrow, of a clonal population of lymphocytes, lymphoplasmacytic cells, and plasma cells, which secrete a monoclonal immunoglobulin (Ig) M [1]. WM corresponds to lymphoplasmacytic lymphoma (LPL) as defined in the Revised European-American Lymphoma (REAL) and World Health Organization classification systems [2, 3]. Most cases of LPL are WM; less than 5% of cases are IgA-secreting, IgG-secreting, or nonsecreting LPL.

Epidemiology

The age-adjusted incidence rate of WM is 3.4 per 1 million among males and 1.7 per 1 million among females in the United States. It increases in incidence geometrically with age [4, 5]. The incidence rate is higher among Americans of European descent. African-American descendants represent approximately 5% of all patients.

Genetic factors play a role in the pathogenesis of WM. Approximately 20% of WM patients are of Ashkenazi-Jewish ethnic background [6]. Familial disease has been reported commonly, including multigenerational clustering of WM and other B-cell lymphoproliferative diseases [6–9]. Approximately 28% of 924 sequential patients with WM presenting to a tertiary referral center had a first- or second-degree relative with either WM or another B-cell disorder [6]. Familial clustering of WM with other immunologic disorders, including hypogammaglobulinemia and hypergammaglobulinemia (particularly polyclonal IgM), autoantibody production (particularly to the thyroid), and manifestation of hyperactive B cells, has also been reported in relatives without WM [6, 9]. Increased expression of the *BCL-2* gene with enhanced survival has been observed in B cells from familial patients and their family members [9].

The role of environmental factors is uncertain, but chronic antigenic stimulation from infections and certain drug or chemical exposures have been considered but have not reached a level of scientific certainty. Hepatitis C virus (HCV) infection was implicated in WM causality in some series, but in a study of 100 consecutive WM patients in whom serologic and molecular diagnostic studies for HCV infection were performed no association was found [10–12].

Pathogenesis

Nature of the WM Clone

Examination of the B-cell clone(s) found in the bone marrow of WM patients reveals a range of differentiation from small lymphocytes with large focal deposits of surface immunoglobulins to lymphoplasmacytic cells and to mature plasma cells that contain intracytoplasmic IgM (Fig. 33.1) [13]. Circulating clonal B cells are often detectable in patients with WM, though lymphocytosis is uncommon [14, 15]. WM cells express the monoclonal IgM, and some clonal cells also express surface IgD [16]. The characteristic immunophenotypic profile of WM lymphoplasmacytic cells includes the expression of the pan B-cell markers CD19, CD20 (including FMC7), CD22, and CD79 [16, 17]. Expression of CD5, CD10, and CD23 can be present in 10–20% of cases, and their presence does not exclude the diagnosis of WM [18]. In addition, multiparameter flow

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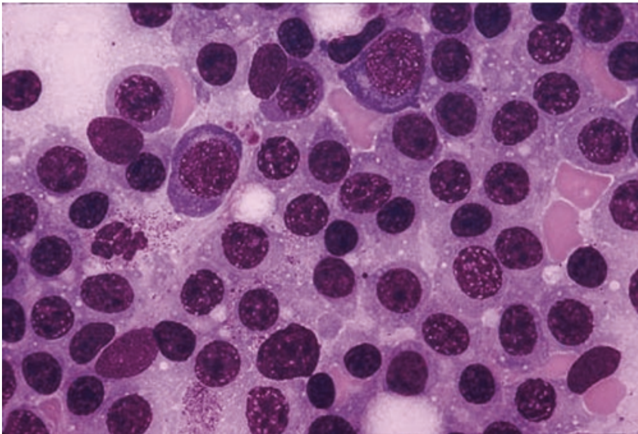

Fig. 33.1 Marrow film from a patient with Waldenstrom's macroglobulinemia. Note infiltrate of mature lymphocytes, lymphoplasmacytic cells, and plasma cells (*used with permission from Marvin J. Stone, MD*)

cytometric analysis has also identified CD25 and CD27 as being characteristic of the WM clone, and that a CD22^{dim}/CD25^{+}/CD27^{+}/IgM^{+} population can be observed among clonal B lymphocytes in IgM MGUS patients who ultimately progressed to WM [19].

Somatic mutations in immunoglobulin genes are present with increased frequency of nonsynonymous versus silent mutations in complement-determining regions along with somatic hypermutation, thereby supporting a post-germinal center derivation for the WM B-cell clone in most patients [20, 21]. A strong preferential usage of VH3/JH4 gene families without intraclonal variation, and without evidence for any isotype-switched transcripts, has also been shown [22, 23]. Taken together, these data support an IgM^{+} and/or IgM^{+}IgD^{+} memory B-cell origin for most cases of WM.

In contrast to myeloma plasma cells, no recurrent translocations have been described in WM, which can help to distinguish IgM myeloma cases that often exhibit t11;14 translocations from WM [24, 25]. Despite the absence of IgH translocations, recurrent chromosomal abnormalities are present in WM cells. These include deletions in chromosome 6q21–23 in 40–60% of WM patients, with concordant gains in 6p in 41% of 6q-deleted patients [26–29]. In a series of 174 untreated WM patients, 6q deletions, followed by trisomy 18, 13q deletions, 17p deletions, trisomy 4, and 11q deletions, were observed [29]. Deletion of 6q and trisomy 4 were associated with adverse prognostic markers in this series. As 6q deletions represent the most recurrent cytogenetic finding in WM cases, there has been great interest in identifying the region of minimal deletion and possible target genes within this region. Two putative gene candidates within this region include TNFAIP3, a negative regulator of nuclear factor kappa B signaling (NFκB), and PRDM1, a master regulator of B-cell differentiation [28, 30]. The removal of an NFκB-negative regulator is of particular interest as the phosphorylation and translocation of NFκB into the nucleus are crucial events for WM cell survival [31]. The success of proteasome inhibitor therapy in WM has been postulated to occur because the degradation of negative regulators of NFκB such as the inhibitor of kappa B (IκB) is blocked [32, 33].

Mutation in MYD88

A highly recurrent somatic mutation (MYD88^{L265P}) was first identified in WM patients by whole-genome sequencing (WGS), and confirmed by multiple studies through Sanger sequencing and/or allele-specific polymerase chain reaction assays [34–39]. MYD88^{L265P} is expressed in 90–95% of WM cases when more sensitive allele-specific PCR has been employed using both CD19-sorted and unsorted bone marrow (BM) cells [35–39]. By comparison, MYD88^{L265P} was absent in myeloma samples, including IgM myeloma, and was expressed in a small subset (6–10%) of MZL patients, who surprisingly have WM-related features [35–37, 40]. By polymerase chain reaction assays, 50–80% of IgM MGUS patients also express MYD88^{L265P}, and expression of this mutation was associated with increased risk for malignant progression [35–37, 41]. The presence of MYD88^{L265P} in IgM MGUS patient suggests a role for this mutation as an early oncogenic driver, and other mutations and/or copy number alterations leading to abnormal gene expression are likely to promote disease progression [28].

The impact of MYD88^{L265P} to growth and survival signaling in WM cells has been addressed in several studies (Fig. 33.2). Knockdown of MYD88 decreased survival of MYD88^{L265P}-expressing WM cells, whereas survival was enhanced by knock-in of MYD88^{L265P} versus wild-type MYD88 [42]. The discovery of a mutation in MYD88 is of significance given its role as an adaptor molecule in Toll-like receptor (TLR) and interleukin-1 receptor (IL-1R) signaling [43]. All TLRs except for TLR3 use MYD88 to facilitate their signaling. Following TLR or IL-1R stimulation, MYD88 is recruited to the activated receptor complex as a homodimer which then complexes with IRAK4 and activates IRAK1 and IRAK2 [44–46]. Tumor necrosis factor receptor-associated factor 6 is then activated by IRAK1 leading to NF-κB activation via IκBα phosphorylation [47]. Use of inhibitors of MYD88 pathway led to decreased IRAK1 and IκBα phosphorylation, as well as survival of MYD88^{L265P}-expressing WM cells. These observations are of particular relevance to WM since NF-κB signaling is important for WM growth and survival [31]. Bruton's tyrosine kinase (BTK) is also activated by MYD88^{L265P} [42]. Activated BTK co-immunoprecipitates with MYD88 that could be abrogated by use of a BTK kinase inhibitor, and overexpression of MYD88^{L265P} but

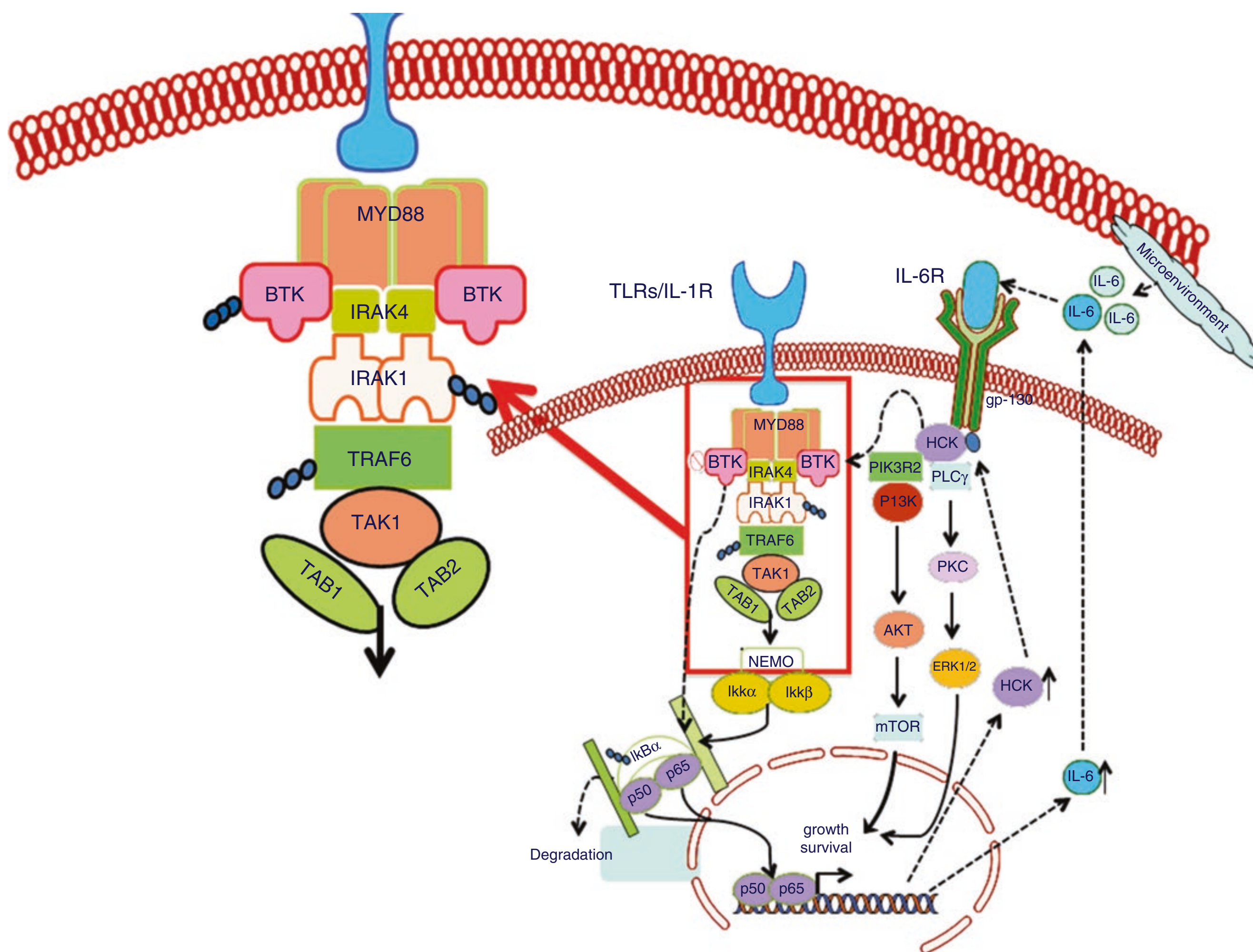

Fig. 33.2 MYD88-activating mutations are highly prevalent in patients with Waldenstrom's macroglobulinemia and trigger multiple growth and survival pathways. Activated MYD88 triggers NF-kB through BTK and IRAK1/IRAK4, as well as HCK that activates BTK, AKT, and ERK

not wild-type (WT) MYD88 triggers BTK activation. Knockdown of MYD88 by lentiviral transfection or use of a MYD88 homodimerization inhibitor also abrogated BTK activation in MYD88^{L265P}-mutated WM cells. MYD88 also triggers HCK, a SRC family member that regulates AKT and ERK survival signaling, and also activates BTK itself [48]. Rarely, non-L265P-activating mutations in WM may also occur, and Sanger sequencing of the entire MYD88 gene should be considered in patients suspected of having WM in whom PCR testing for MYD88^{L265P} is negative [49].

CXCR4 WHIM Mutations

The second most common somatic mutation after MYD88^{L265P} revealed by whole-genome sequencing was found in the C-terminus of the CXCR4 receptor. These mutations are present in 30–35% of WM patients, and impact serine phosphorylation sites that regulate CXCR4 signaling by its only known ligand SDF-1a (CXCL12) [28, 50–52]. The location of somatic mutations found in the C-terminus of CXCR4 in WM is similar to that observed in the germline of patients with WHIM (warts, hypogammaglobulinemia, infections, and myelokathexis) syndrome, a congenital immunodeficiency disorder characterized by chronic noncyclic neutropenia [53]. Patients with WHIM syndrome exhibit impaired CXCR4 receptor internalization following SDF-1a stimulation, which results in persistent CXCR4 activation and myelokathexis [54].

In WM patients, two classes of CXCR4 mutations occur in the C-terminus. These include non-sense (CXCR4$^{WHIM/NS}$) mutations that truncate the distal 15–20 amino acid region, and frameshift (CXCR4$^{WHIM/FS}$) mutations that compromise a region of up to 40 amino acids in the C- terminal domain [28, 50]. Non-sense and frameshift mutations are almost equally divided among WM patients with CXCR4 somatic mutations, and over 30 different types of CXCR4^{WHIM} mutations have been identified in WM patients [28, 50]. In some

patients multiple $CXCR^{WHIM}$ mutations may be detected. $CXCR4^{WHIM}$ mutations are usually subclonal to MYD88, with highly variable clonal distribution [55]. The subclonal nature of these mutations suggests that CXCR4 mutations were likely acquired after MYD88 mutations.

Preclinical studies with WM cells engineered to express nonsense and frameshift $CXCR4^{WHIM}$-mutated receptors have shown enhanced and sustained AKT and ERK signaling following SDF-1a relative to $CXCR4^{WT}$, as well as increased cell migration, adhesion, growth and survival, and drug resistance (including ibrutinib) in WM cells [56, 57].

Other Somatic Events

Many copy number alterations have been revealed in WM patients that impact growth and survival pathways. Frequent loss of HIVEP2 (80%) and TNAIP3 (50%) genes that are negative regulators of NFkB expression, as well as LYN (70%) and IBTK (40%) that modulate BCR signaling, has been revealed by WGS [28]. WGS has also revealed common defects in chromatin remodeling with somatic mutations in ARID1A present in 17%, and loss of ARID1B in 70% of WM patients. Both ARID1A and ARID1B are members of the SWI/SNF family of proteins, and are thought to exert their effects via p53 and CDKN1A regulation. TP53 is mutated in 7% of sequenced WM genomes, while PRDM2 and TOP1 that participate in TP53-related signaling are deleted in 80% and 60% of WM patients, respectively [28]. Taken together, somatic events that contribute to impaired DNA damage response are also common in WM.

Impact of WM Genomics on Clinical Presentation

The importance of MYD88 and CXCR4 mutations in the clinical presentation of WM patients was recently reported. Significantly higher BM disease involvement, serum IgM levels, and symptomatic disease requiring therapy, including hyperviscosity syndrome, were observed in those patients with $MYD88^{L265P}CXCR4^{WHIM/NS}$ mutations [50]. Patients with $MYD88^{L265P}CXCR4^{WHIM/FS}$ or $MYD88^{L265P}CXCR4^{WT}$ had intermediate BM and serum IgM levels; those with $MYD88^{WT}CXCR4^{WT}$ showed the lowest BM disease burden. Fewer patients with $MYD88^{L265P}$ and $CXCR4^{WHIM/FS\ or\ NS}$ compared to $MYD88^{L265P}CXCR4^{WT}$ presented with adenopathy, further delineating differences in disease tropism based on CXCR4 status. Despite the more aggressive presentation associated with $CXCR4^{WHIM/NS}$ genotype, risk of death was not impacted by CXCR4 mutation status. Risk of death was found to be tenfold higher in patients with $MYD88^{WT}$ versus $MYD88^{L265P}$ genotype [50].

Marrow Microenvironment

Increased numbers of mast cells are found in the bone marrow of WM patients, wherein they are usually admixed with tumor cell aggregates (Fig. 33.3) [13, 17, 55]. The role of mast cells in WM has been investigated in one study wherein coculture of primary autologous or mast cell lines with WM LPC resulted in dose-dependent WM cell proliferation and/or tumor colony formation, through CD40 ligand (CD40L) signaling [58]. WM cells release soluble CD27 (sCD27) which may be triggered by cleavage of membrane-bound CD27 by matrix metalloproteinase 8 (MMP8) [59]. sCD27 levels are elevated in the serum of WM patients, and follow disease burden in mice engrafted with WM cells, as well as in WM patients [61]. sCD27 triggers the upregulation of CD40L as well as a proliferation-inducing ligand (APRIL) on mast cells derived from WM patients, as well as mast cell lines through its receptor CD70. Modeling in mice engrafted with a CD70-blocking antibody shows inhibition of tumor cell growth suggesting that WM cells require a microenvironmental support system for their growth and survival [60]. High levels of CXCR4 and very late antigen-4 (VLA-4) have also been observed in WM cells [61]. In blocking experiments studies, CXCR4 was shown to support migration of WM cells, while VLA-4 contributed to adhesion of WM cells to bone marrow stromal cells [61].

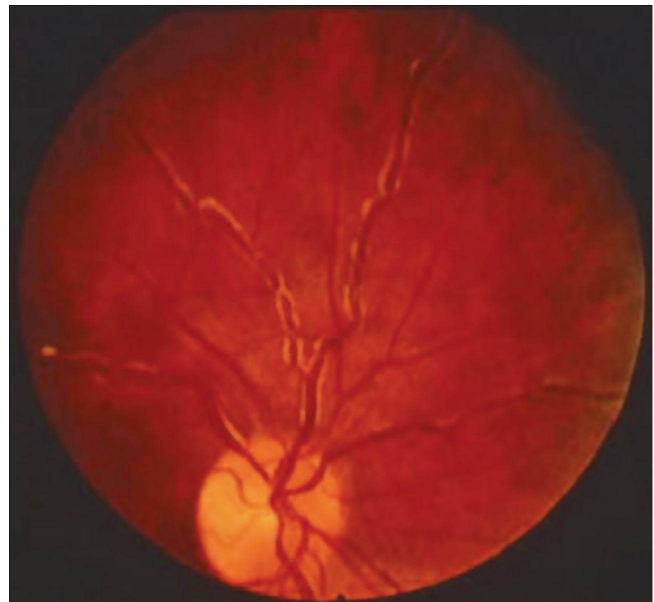

Fig. 33.3 Funduscopic examination of a patient with Waldenstrom's macroglobulinemia with hyperviscosity-related changes, including dilated retinal vessels, hemorrhages, and "venous sausaging." The white material at the edge of the veins may be cryoglobulin (*used with permission from Marvin J. Stone, MD*)

Clinical Features

Table 33.1 presents the clinical and laboratory findings at the time of diagnosis of WM in one large institutional study [15]. Unlike most indolent lymphomas, splenomegaly and lymphadenopathy are uncommon (≤15%). Purpura is frequently associated with cryoglobulinemia and in rare circumstances with light-chain (AL) amyloidosis. Hemorrhagic and neuropathic manifestations are multifactorial (see "IgM-Related Neuropathy" below). The morbidity associated with WM is caused by the concurrence of two main components: tissue infiltration by neoplastic cells and, importantly, the physicochemical and immunologic properties of the monoclonal IgM. As shown in Table 33.2, the monoclonal IgM can produce clinical manifestations through several different mechanisms related to its physicochemical properties, nonspecific interactions with other proteins, antibody activity, and tendency to deposit in tissues [62–64].

Morbidity Mediated by the Effects of IGM

Hyperviscosity Syndrome

The increased plasma IgM levels lead to blood hyperviscosity and its complications [65]. The mechanisms behind the marked increase in the resistance to blood flow and the resulting impaired transit through the microcirculatory system are complex [65–68]. The main determinants are (1) a high concentration of monoclonal IgMs, which may form aggregates and may bind water through their carbohydrate component, and (2) their interaction with blood cells. Monoclonal IgM increases red cell aggregation (rouleaux formation) and red cell internal viscosity

Table 33.1. Clinical and laboratory findings for 356 consecutive newly diagnosed patients with Waldenstrom's macroglobulinemia [14]

	Median	Range	Normal reference range
Age (years)	58	32–91	NA
Gender (male/female)	215/141		NA
Marrow involvement (% of area on slide)	30	5–95	NA
Adenopathy (% of patients)	15		NA
Splenomegaly (% of patients)	10		NA
IgM (mg/dL)	2620	270–12,400	40–230
IgG (mg/dL)	674	80–2770	700–1600
IgA (mg/dL)	58	6–438	70–400
Serum viscosity (cp)	2.0	1.1–7.2	1.4–1.9
Hematocrit (%)	35	17–45	35–44
Platelet count (×10^9/L)	275	42–675	155–410
White cell count (×10^9/L)	6.4	1.7–22	3.8–9.2
β_2-M (mg/dL)	2.5	0.9–13.7	0–2.7
LDH (U/mL)	313	61–1701	313–618

β_2M β_2-microglobulin, *cp* centipoise, *LDH* lactic dehydrogenase, *NA* not applicable
Source: Data from patients seen at the Dana Farber Cancer Institute, Boston, MA

Table 33.2 Physicochemical and immunological properties of the monoclonal IGM protein in Waldenstrom's macroglobulinemia [62–64]

Properties of IgM monoclonal protein	Diagnostic condition	Clinical manifestations
Pentameric structure	Hyperviscosity	Headaches, blurred vision, epistaxis, retinal hemorrhages, leg cramps, impaired mentation, intracranial hemorrhage
Precipitation on cooling	Cryoglobulinemia (type I)	Raynaud phenomenon, acrocyanosis, ulcers, purpura, cold urticaria
Autoantibody activity to myelin-associated glycoprotein, ganglioside M_1, sulfatide moieties on peripheral nerve sheaths	Peripheral neuropathies	Sensorimotor neuropathies, painful neuropathies, ataxic gait, bilateral foot drop
Autoantibody activity to IgG	Cryoglobulinemia (type II)	Purpura, arthralgia, renal failure, sensorimotor neuropathies
Autoantibody activity to red blood cell antigens	Cold agglutinins	Hemolytic anemia, Raynaud phenomenon, acrocyanosis, livedo reticularis
Tissue deposition as amorphous aggregates	Organ dysfunction	Skin: Bullous skin disease, papules, Schnitzler syndrome
		Gastrointestinal: Diarrhea, malabsorption, bleeding
		Kidney: Proteinuria, renal failure (light-chain component)
Tissue deposition as amyloid fibrils (light-chain component most commonly)	Organ dysfunction	Fatigue, weight loss, edema, hepatomegaly, macroglossia, organ dysfunction of involved organs (heart, kidney, liver, peripheral sensory, and autonomic nerves)

while reducing red cell deformability. The presence of cryoglobulins contributes to increasing blood viscosity, as well as to the tendency to induce erythrocyte aggregation. Serum viscosity is proportional to IgM concentration up to 30 g/L, and then increases sharply at higher levels. Increased plasma viscosity may also contribute to inappropriately low erythropoietin production, which is the major reason for anemia in these patients [68]. Renal synthesis of erythropoietin is inversely correlated with plasma viscosity. Clinical manifestations are related to circulatory disturbances that can be best appreciated by ophthalmoscopy, which shows distended and tortuous retinal veins, hemorrhages, and papilledema (Fig. 33.4) [69]. Symptoms usually occur when the monoclonal IgM concentration exceeds 50 g/L or when serum viscosity is >4.0 centipoises (cp), but there is individual variability, with some patients showing no evidence of hyperviscosity even at 10 cp [65]. The most common symptoms are oronasal mucosal bleeding, visual disturbances because of retinal bleeding, and dizziness that rarely may lead to stupor or coma. Heart failure can be aggravated, particularly in the elderly, owing to increased blood viscosity, expanded plasma volume, and anemia. Inappropriate red cell transfusion can exacerbate hyperviscosity and may precipitate cardiac failure.

Cryoglobulinemia

The monoclonal IgM can behave as a cryoglobulin in up to 20% of patients, and is usually type I and asymptomatic in most cases [15, 65, 70]. Cryoprecipitation is mainly dependent on the concentration of monoclonal IgM; for this reason plasmapheresis or plasma exchange is commonly effective in this condition. Symptoms result from impaired blood flow in small vessels and include Raynaud phenomenon, acrocyanosis, and necrosis of the regions most exposed to cold, such as the tip of the nose, ears, fingers, and toes (Fig. 33.5); malleolar ulcers; purpura; and cold urticaria. Renal manifestations are infrequent. Mixed cryoglobulins (type II) consisting of IgM-IgG complexes may be associated with hepatitis C infections [70].

Autoantibody Activity

Monoclonal IgM may exert its pathogenic effects through specific recognition of autologous antigens, the most notable being nerve constituents, immunoglobulin determinants, and red blood cell antigens.

IgM-Related Neuropathy

IgM-related peripheral neuropathy is common in WM patients, with estimated prevalence rates of 5–40% [71–73]. Approximately 8% of idiopathic neuropathies are associated with a monoclonal gammopathy, with a preponderance of IgM (60%) followed by IgG (30%) and IgA (10%) [74, 75]. The nerve damage is mediated by diverse pathogenetic mechanisms: (1) IgM antibody activity toward nerve constituents causing demyelinating polyneuropathies; (2) endoneurial granulofibrillar deposits of IgM without antibody activity, associated with axonal polyneuropathy; (3) occasionally by tubular deposits in the endoneurium associated with IgM cryoglobulin; and, rarely, (4) by amyloid deposits or by neoplastic cell infiltration of nerve structures [73, 76].

Half of the patients with IgM neuropathy have a distinctive clinical syndrome that is associated with antibodies against a minor 100-kDa glycoprotein component of nerve known as the myelin-associated glycoprotein (MAG). Anti-MAG antibodies are generally monoclonal IgMκ, and usually also exhibit reactivity with other glycoproteins or glycolipids that share antigenic determinants with MAG [77–79]. The anti–MAG-related neuropathy is typically distal and symmetrical,

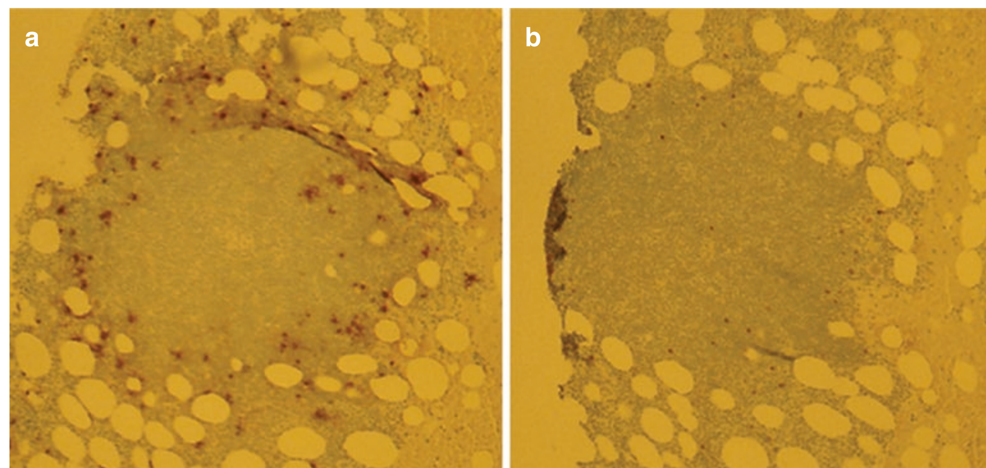

Fig. 33.4 Cryoglobulinemia manifesting with severe acrocyanosis in a patient with Waldenstrom's macroglobulinemia before (**a**) and following warming and plasmapheresis (**b**)

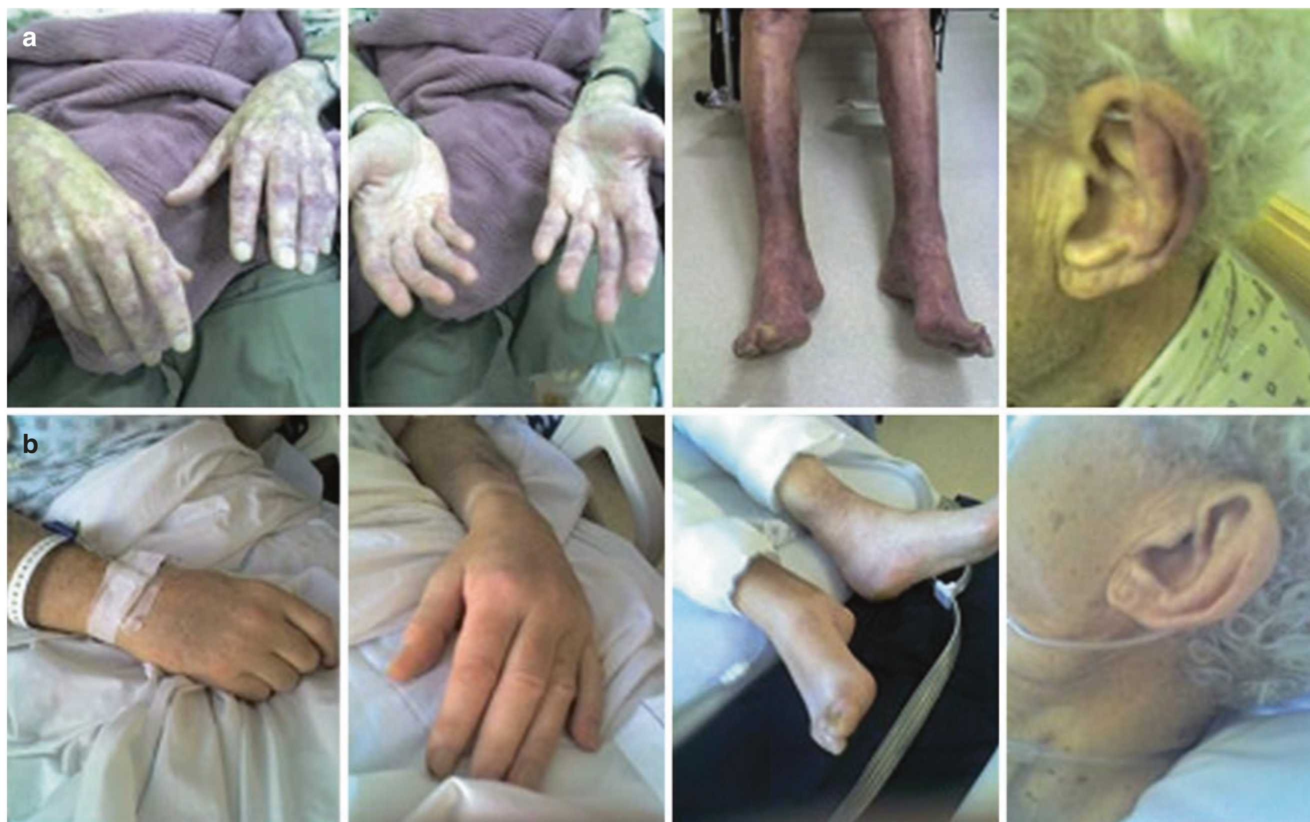

Fig. 33.5 Marrow clot section. (**a**) Tryptase-staining mast cells surrounding a nodule of lymphoplasmacytic cells in a patient with Waldenstrom's macroglobulinemia. (**b**) Mast cells in the same section exhibit strong CD40 ligand signaling, which has been shown to support (at least in part) the growth and survival of lymphoplasmacytic cells

affecting both motor and sensory functions; it is slowly progressive with a long period of stability [72, 80]. Most patients present with sensory complaints (paresthesias, aching discomfort, dysesthesias, or lancinating pains), imbalance, and gait ataxia, owing to lack of proprioception and leg muscle atrophy in advanced stage. Patients with predominantly demyelinating sensory neuropathy in association with monoclonal IgM to gangliosides with disialosyl moieties, such as GD1b, GD3, GD2, GT1b, and GQ1b have also been reported [81, 82]. Anti-GD1b and anti-GQ1b antibodies were associated with sensory ataxic neuropathy. These antiganglioside monoclonal IgMs present core clinical features of chronic ataxic neuropathy sometimes with ophthalmoplegia and/or red blood cell cold-agglutinating activity. The disialosyl epitope is also present on red blood cell glycophorins, thereby accounting for the red cell cold agglutinin activity of anti-Pr2 specificity [83, 84]. Monoclonal IgM proteins that bind to gangliosides with a terminal trisaccharide moiety, including ganglioside M_2 (GM_2) and GalNac-GD1A, are associated with chronic demyelinating neuropathy and severe sensory ataxia, unresponsive to glucocorticoids [85]. Antiganglioside IgM proteins may also cross-react with lipopolysaccharides of *Campylobacter jejuni*, whose infection is known to precipitate the Miller-Fisher syndrome, a variant of the Guillain-Barré syndrome [86]. Thus, molecular mimicry may play a role in this condition. Antisulfatide monoclonal IgM proteins, associated with sensory-sensorimotor neuropathy, have been detected in 5% of patients with IgM monoclonal gammopathy and neuropathy [87]. Motor neuron disease has been reported in patients with WM and monoclonal IgM with anti-GM_1 and sulfoglucuronyl paragloboside activity [88]. Polyneuropathy, organomegaly, endocrinopathy, M-protein, and skin changes (the POEMS syndrome) are rare in patients with WM [89].

Cold Agglutinin Hemolytic Anemia

Monoclonal IgM may have cold agglutinin activity; that is, it can recognize specific red cell antigens at temperatures below 37 °C, producing chronic hemolytic anemia. This disorder occurs in <10% of WM patients and is associated with cold agglutinin titers greater than 1:1000 in most cases [90]. The monoclonal component is usually an IgMκ and reacts most commonly with red cell I/i antigens, resulting in complement fixation and activation [91, 92]. Mild-to-moderate

chronic hemolytic anemia can be exacerbated after cold exposure. Hemoglobin usually remains above 70 g/L. The hemolysis is usually extravascular, mediated by removal of C3b-opsonized red cells by the mononuclear phagocyte system, primarily in the liver. Intravascular hemolysis from complement destruction of red blood cell membrane is infrequent. The agglutination of red cells in the skin circulation also causes Raynaud syndrome, acrocyanosis, and livedo reticularis. Macroglobulins with the properties of both cryoglobulins and cold agglutinins with anti-Pr specificity can occur. These properties may have as a common basis the binding of the sialic acid-containing carbohydrate present on red blood cell glycophorins and on Ig molecules. Several other macroglobulins with antibody activity toward autologous antigens (i.e., phospholipids, tissue and plasma proteins) and foreign ligands have also been described.

IgM Tissue Deposition

The monoclonal protein can deposit in several tissues as amorphous aggregates. Linear deposition of monoclonal IgM along the skin basement membrane is associated with bullous skin disease [93]. Amorphous IgM deposits in the dermis result in IgM storage papules on the extensor surface of the extremities, referred to as macroglobulinemia cutis [94]. Deposition of monoclonal IgM in the lamina propria and/or submucosa of the intestine may be associated with diarrhea, malabsorption, and gastrointestinal bleeding [95, 96]. Kidney involvement is less common and less severe in WM than in myeloma, probably because the amount of light chain excreted in the urine is generally lower in WM than in myeloma and because of the absence of contributing factors, such as hypercalcemia. Urinary cast nephropathy, however, has occurred in WM [97]. On the other hand, the IgM macromolecule is more susceptible to being trapped in the glomerular loops where ultrafiltration presumably contributes to its precipitation, forming subendothelial deposits of aggregated IgM proteins that occlude the glomerular capillaries [98]. Mild and reversible proteinuria may result and most patients are asymptomatic. The deposition of monoclonal light chain as fibrillar amyloid deposits (AL amyloidosis) is uncommon in patients with WM [99]. Clinical expression and prognosis are similar to those of other AL amyloidosis patients with involvement of heart (44%), kidneys (32%), liver (14%), lungs (10%), peripheral or autonomic nerves (38%), and soft tissues (18%). The incidence of cardiac and pulmonary involvement is higher in patients with monoclonal IgM than with other immunoglobulin isotypes. The association of WM with reactive amyloidosis has been documented rarely [100, 101]. Simultaneous occurrence of fibrillary glomerulopathy, characterized by glomerular deposits of wide noncongophilic fibrils and amyloid deposits, has been described [102].

Manifestations Related to Tissue Infiltration by Neoplastic Cells

Tissue infiltration by neoplastic cells is uncommon but can involve various organs and tissues, including the liver, spleen, lymph nodes, lungs, gastrointestinal tract, kidneys, skin, eyes, and central nervous system.

Lung

Pulmonary involvement in the form of masses, nodules, diffuse infiltrate, or pleural effusions is uncommon; the overall incidence of pulmonary and pleural findings is approximately 4% [103–105]. Cough is the most common presenting symptom, followed by dyspnea and chest pain. Chest radiographic findings include parenchymal infiltrates, confluent masses, and effusions.

Gastrointestinal Tract

Malabsorption, diarrhea, bleeding, or obstruction may indicate involvement of the gastrointestinal tract at the level of the stomach, duodenum, or small intestine [106–109].

Renal System

In contrast to myeloma, infiltration of the kidney interstitium with lymphoplasmacytoid cell can occur in WM, and renal or perirenal masses are not uncommon [110, 111].

Skin

The skin can be the site of dense lymphoplasmacytic infiltrates, similar to those seen in the liver, spleen, and lymph nodes, forming cutaneous plaques and, rarely, nodules [112]. Chronic urticaria and IgM gammopathy are the two cardinal features of the Schnitzler syndrome, which is not usually associated initially with clinical features of WM, although evolution to WM is not uncommon [113]. Thus, close follow-up of these patients is important.

Joints

Invasion of articular and periarticular structures by WM malignant cells is rarely reported [114].

Eye

The neoplastic cells can infiltrate the periorbital structures, lacrimal gland, and retro-orbital lymphoid tissues, resulting in ocular nerve palsies [115, 116].

Central Nervous System

Direct infiltration of the central nervous system by monoclonal lymphoplasmacytic cells as infiltrates or as tumors constitutes the rarely observed Bing-Neel syndrome, characterized clinically by confusion, memory loss, disorientation, and motor dysfunction. The diagnosis and management of Bing-Neel syndrome are reviewed in 117.

Laboratory Findings

Blood Abnormalities

Anemia is the most common finding in patients with symptomatic WM and is caused by a combination of factors: decrease in red cell survival, impaired erythropoiesis, moderate plasma volume expansion, hepcidin production leading to iron reutilization defect, and blood loss from the gastrointestinal tract [15, 118, 119]. Blood films are usually normocytic and normochromic, and rouleaux formation is often pronounced. Mean red cell volume may be elevated spuriously owing to erythrocyte aggregation. In addition, the hemoglobin estimate can be inaccurate, that is, falsely high, because of interaction between the monoclonal protein and the diluent used in some automated analyzers [120]. Leukocyte and platelet counts are usually within the reference range at presentation, although patients may occasionally present with severe thrombocytopenia. Monoclonal B-lymphocytes expressing surface IgM and late-differentiation B-cell markers are uncommonly detected in blood by flow cytometry. A raised erythrocyte sedimentation rate is almost always present and may be the first clue to the presence of the macroglobulinemia. The clotting abnormality detected most frequently is prolongation of thrombin time. AL amyloidosis should be suspected in all patients with nephrotic syndrome, cardiomyopathy, hepatomegaly, or peripheral neuropathy. Diagnosis requires the demonstration of green birefringence under polarized light of amyloid deposits stained with Congo red.

Marrow Findings

Central to the diagnosis of WM is the demonstration, by trephine biopsy, of marrow infiltration by a lymphoplasmacytic cell population characterized by small lymphocytes with evidence of plasmacytoid and plasma cell maturation (Fig. 33.1) [1, 13]. The pattern of marrow infiltration may be diffuse, interstitial, or nodular, usually with an intertrabecular pattern of infiltration. A solely paratrabecular pattern of infiltration is unusual and should raise the possibility of follicular lymphoma [1]. The marrow cell immunophenotype should be confirmed by flow cytometry and/or immunohistochemistry. The cell immunoprofile sIgM^{+}CD19^{+}CD20^{+}CD22^{+}CD79^{+} is characteristic of WM [13, 120, 121]. Up to 20% of cases may express either CD5, CD10, or CD23 [18]. In these cases, chronic lymphocytic leukemia and mantle cell lymphoma should be excluded. "Intranuclear" periodic acid-Schiff–positive inclusions (Dutcher-Fahey bodies) [122] consisting of IgM deposits in the perinuclear space, and sometimes in intranuclear vacuoles, may be seen occasionally in lymphoid cells. An increased number of mast cells, usually in association with the lymphoid aggregates, is commonly found, and their presence may help in differentiating WM from other B-cell lymphomas (see Fig. 33.3) [13]. MYD88^{L265P} testing of bone marrow samples has been incorporated into many clinical laboratories, and may help in clarifying the diagnosis of WM from other IgM-secreting entities [34–38]. The use of peripheral blood B cells may also permit determination of MYD88^{L265P} status by allele-specific polymerase chain reaction assays, particularly in untreated WM patients. CXCR4 mutation testing may also be useful in patients being considered for ibrutinib therapy (discussed below).

Immunologic Abnormalities

High-resolution electrophoresis combined with immunofixation of serum and urine is recommended for identification and characterization of the IgM monoclonal protein. The light chain of the monoclonal IgM is κ in 75–80% of patients. More than one M-component may be present. The concentration of the serum monoclonal protein is very variable but in most cases lies within the range of 15–45 g/L. Densitometry should be adopted to determine IgM levels for serial evaluations because nephelometry is unreliable and shows large laboratory variation. The presence of cold agglutinins or cryoglobulins may affect determination of IgM levels and, therefore, testing for cold agglutinins and cryoglobulins should be performed at diagnosis. If present, subsequent serum samples should be analyzed at 37 °C for determination of serum monoclonal IgM level. Although Bence Jones proteinuria is frequently present, it exceeds 1 g/24 h in only 3% of cases. Whereas IgM levels are elevated in WM patients, IgA and IgG levels are most often depressed and do not recover after successful treatment [123].

Serum Viscosity

Because of its large size (almost 1,000,000 daltons), most IgM molecules are retained within the intravascular compartment and can exert an undue effect on serum viscosity [65]. Serum viscosity can be measured if the patient has signs or symptoms of hyperviscosity syndrome, though levels often slow to be resulted and erratic due to a lack of standardization in many clinical laboratories [15]. As such, serum IgM levels may be more expedient and relied upon. Patients typically become symptomatic at serum viscosity levels of 4.0 centipoise and above that relates to serum IgM levels above 6000 mg/dL [124, 125]. Patients may be symptomatic at lower serum viscosity and IgM levels, and in these patients cryoglobulins may be present. Recurring nosebleeds, headaches, and visual disturbances are common symptoms in patients with symptomatic hyperviscosity [15]. Funduscopy is an important indicator of clinically relevant hyperviscosity. Among the first clinical signs of hyperviscosity are the appearance of peripheral and midperipheral dot- and blot-like hemorrhages in the retina, which are best appreciated with indirect ophthalmoscopy and scleral depression [69]. In more severe cases of hyperviscosity, dot-, blot-, and flame-shaped hemorrhages can appear in the macular area along with markedly dilated and tortuous veins with focal constrictions resulting in "venous sausaging," as well as papilledema (Fig. 33.4).

Imaging

Magnetic resonance imaging (MRI) of the spine in conjunction with computed tomography (CT) of the abdomen and pelvis is useful in evaluating the disease status [126]. Marrow involvement can be documented by MRI studies of the spine in more than 90% of patients; CT of the abdomen and pelvis demonstrates enlarged nodes in approximately 20% of WM patients at diagnosis but may be higher at relapse [126].

Lymph Node Biopsy

Lymph node biopsy may show preserved architecture or replacement by infiltration of neoplastic cells with lymphoplasmacytoid, lymphoplasmacytic, or polymorphous cytologic patterns. Testing for MYD88 mutations may help.

Treatment

Initiating Treatment

As part of the Second International Workshop on Waldenstrom's macroglobulinemia, a consensus panel was organized to recommend criteria for the initiation of therapy in patients with WM [127]. The panel recommended that initiation of therapy should not be based on the IgM level per se, as this may not correlate with the clinical manifestations of WM. The consensus panel did, however, agree that initiation of therapy is appropriate for patients with constitutional symptoms, such as recurrent fever, night sweats, fatigue as a consequence of anemia, or weight loss. Progressive symptomatic lymphadenopathy and/or splenomegaly provide additional reasons to begin therapy. Anemia with a hemoglobin value of ≤10 g/dL or a platelet count of ≤100 × 10^9/L owing to marrow infiltration also justifies treatment. Certain complications, such as hyperviscosity syndrome, symptomatic sensorimotor peripheral neuropathy, systemic amyloidosis, renal insufficiency, or symptomatic cryoglobulinemia, may also be indications for therapy [15, 127].

Initial Therapy

The International Workshops on Waldenström's Macroglobulinemia have also formulated consensus recommendations for both initial therapy and therapy for refractory disease based on the best available evidence. The most recent recommendations emerged from the Eighth International Workshop on WM [128]. Individual patient considerations, including the presence of cytopenias, need for more rapid disease control, age, and candidacy for autologous transplant therapy, should be taken into account in making the choice of the drugs to use. For patients who are candidates for autologous stem cell transplantation, which typically is reserved for those patients younger than 70 years of age, the panel recommended that exposure to alkylating agents or nucleoside analogues should be limited. The use of nucleoside analogues should be approached cautiously in WM patients as there appears to be an increased risk for the development of disease transformation as well as myelodysplasia and acute myelogenous leukemia.

Oral Alkylating Agents

Oral alkylating drugs, alone and in combination therapy with glucocorticoids, have been extensively evaluated in the treatment of WM. Chlorambucil has been administered on both a continuous (i.e., daily-dose schedule) and an intermittent schedule. Patients receiving chlorambucil on a continuous schedule typically receive 0.1 mg/kg per day, whereas on the intermittent schedule patients typically receive 0.3 mg/kg for 7 days, every 6 weeks. In a prospective randomized study, no significant difference in the overall response rate between these schedules was observed [129], although the median response duration was greater for patients receiving intermittent- versus continuous-dose chlorambucil (46 versus 26 months). Despite the favorable median response duration in this study for use of the

intermittent schedule, no difference in the median overall survival was observed. Moreover, an increased incidence for development of myelodysplasia and acute myelogenous leukemia with the intermittent (3 of 22 patients) versus the continuous (0 of 24 patients) chlorambucil schedule prompted the preference for use of continuous chlorambucil dosing. The use of glucocorticoids in combination with alkylating agent therapy has also been explored. Chlorambucil (8 mg/m^2) plus prednisone (40 mg/m^2) given orally for 10 days, every 6 weeks, resulted in a major response (i.e., reduction of IgM by more than 50%) in 72% of patients [130]. Alkylating agent regimens employing melphalan and cyclophosphamide in combination with glucocorticoids have also been examined [131, 132]. This approach produced slightly higher overall response rates and response durations, although the benefit of these more complex regimens over chlorambucil remains to be demonstrated. Pretreatment factors associated with shorter survival in the entire population of patients receiving single-agent chlorambucil were age older than 60 years, male sex, hemoglobin less than 10 g/dL, leukocytes less than 4×10^9/L, and platelets less than 150×10^9/L. Organomegaly, signs of hyperviscosity, renal failure, monoclonal IgM level, blood lymphocytosis, and percentage of marrow lymphoid cells were not significantly correlated with survival [133]. Additional factors to be taken into account in considering alkylating agent therapy for patients with WM include necessity for more rapid disease control given the slow response, as well as consideration for preserving stem cells in patients who are candidates for autologous stem cell transplantation therapy. A large randomized study showed an inferior response rate and time to progression in WM patients receiving chlorambucil versus fludarabine, as well as a higher incidence of secondary malignancies in the former. Neutropenia was however more pronounced in those patients on fludarabine [134].

Nucleoside Analogue Therapy

Cladribine administered as a single agent by continuous intravenous infusion, by 2-h daily infusion, or by subcutaneous bolus injections for 5–7 days has resulted in major responses in 40–90% of patients who received primary therapy, whereas in the previously treated patients, responses have ranged from 38 to 54% [135–141]. Median time to achievement of response in responding patients following cladribine ranged from 1.2 to 5 months. The overall response rate with daily infusion of fludarabine, administered mainly on 5-day schedules, in previously untreated and treated patients ranged from 38 to 100% and 30 to 40%, respectively [142–147], similar to the responses to cladribine. Median time to achievement of response for fludarabine (3–6 months) was also similar to cladribine. In general, response rates and durations of responses have been greater for patients receiving nucleoside analogues as initial therapy, although in several studies in which both untreated and previously treated patients were enrolled, no difference in the overall response rate was reported.

Myelosuppression commonly occurs following prolonged exposure to either of the nucleoside analogues. A sustained decrease in both CD4+ and CD8+ T lymphocytes, measured 1 year following initiation of therapy, is notable [135–137]. Treatment-related mortality as a consequence of myelosuppression and/or opportunistic infections attributable to immunosuppression occurred in up to 5% of all treated patients in some series with nucleoside analogues.

Factors predicting for a better response to nucleoside analogues include younger age at start of treatment (<70 years), higher pretreatment hemoglobin (>95 g/L), higher platelet count ($>75 \times 10^9$/L), disease relapsing off therapy, and a long interval between first-line therapy and initiation of a nucleoside analogue in relapsing patients [135, 140, 146]. There are limited data on the use of an alternate nucleoside analogue in previously treated patients among whom disease relapsed or who had resistance when not on cladribine or fludarabine therapy [148, 149]. Three of four (75%) patients responded to cladribine after progression following an unmaintained remission to fludarabine, whereas only one of ten (10%) with disease resistant to fludarabine responded to cladribine [148]. A response in two of six patients (33%) and disease stabilization in the remaining patients to fludarabine, in spite of an inadequate response or progressive disease, following cladribine therapy has been reported [149].

Harvesting autologous blood stem cells succeeded on the first attempt in 14 of 15 patients who did not receive nucleoside analogue therapy as compared to 2 of 6 patients who received a nucleoside analogue [150]. A sevenfold increase in transformation to an aggressive lymphoma and a threefold increase in the development of myelodysplasia or acute myelogenous leukemia were observed among patients who received a nucleoside analogue versus other therapies for their WM [151]. A meta-analysis of several trials in which patients were treated with nucleoside analogues in WM patients, included patients who had previously received an alkylating agent, and showed a crude incidence of approximately 8% for development of disease transformation and of approximately 5% for development of myelodysplasia or acute myelogenous leukemia [152]. None of the risk factors—that is, gender, age, family history of WM, or B-cell malignancies, typical markers of tumor burden and prognosis, type of nucleoside analogue therapy (cladribine versus fludarabine), time from diagnosis to nucleoside analogue use, nucleoside analogue treatment as primary or salvage therapy, or treatment with an oral alkylator (i.e., chlorambucil)—predicted for the occurrence of transformation or development of myelodysplasia or acute myelogenous leukemia in patients treated with a nucleoside analogue [152].

CD20-Directed Antibody Therapy

Rituximab is a chimeric monoclonal antibody that targets CD20, a widely expressed antigen on lymphoplasmacytic cells in WM [153]. Several retrospective and prospective studies have indicated that rituximab, when used at standard doses (i.e., 4-weekly infusions of 375 mg/m^2), induced major responses in approximately 30% of previously treated and untreated patients [154, 155]. Even patients who achieved minor responses benefited from rituximab by improved hemoglobin and platelet counts, and reduction of lymphadenopathy and/or splenomegaly [154]. The median time to treatment failure in these studies was found to range from 8 to 27+ months. Patients on an extended rituximab schedule consisting of 4-weekly courses at 375 mg/m^2 per week, repeated 3 months later by another 4-week course, have demonstrated major response rates of approximately 45%, with time to progression estimates of 16+ to 29+ months [156, 157].

In many WM patients, a transient increase or flare of the serum IgM may occur immediately following initiation of rituximab treatment [156, 158, 159]. Such an increase does not herald treatment failure and most patients will return to their baseline serum IgM level by 12 weeks. Some patients continue to show a prolonged increase in IgM despite an apparent reduction in their marrow tumor cells. However, patients with baseline serum IgM levels of >50 g/dL or serum viscosity of >3.5 cp may be particularly at risk for a hyperviscosity-related event and plasmapheresis should be considered in these patients in advance of rituximab therapy [158]. Because of the decreased likelihood of response in patients with higher IgM levels, as well as the possibility that serum IgM and blood viscosity levels may abruptly rise, rituximab monotherapy should not be used as sole therapy for the treatment of patients at risk for hyperviscosity symptoms [128, 156, 157].

Time to response after rituximab is slow and exceeds 3 months on the average. The time to best response in one study was 18 months [157]. Patients with baseline serum IgM levels of <60 g/dL are more likely to respond, regardless of the underlying marrow involvement by tumor cells [156, 157]. An analysis of 52 patients who were treated with single-agent rituximab found that the objective response rate was significantly lower in patients who had either low serum albumin (<35 g/L) or a serum monoclonal protein greater than 40 g/L. [160] The presence of both adverse prognostic factors was associated with a short time to progression (3.6 months). Patients who had normal serum albumin and relatively low serum monoclonal protein levels derived a substantial benefit from rituximab with a time to progression exceeding 40 months.

A correlation between polymorphisms at position 158 in the FcγRIIIa receptor (CD16), an activating Fc receptor on important effector cells that mediate antibody-dependent cell-mediated cytotoxicity, and rituximab response was observed in WM patients [161]. Individuals may encode either the amino acid valine or phenylalanine at position 158 in the FcγRIIIa receptor. WM patients who carried the valine amino acid (either in a homozygous or in a heterozygous pattern) had a fourfold higher major response rate (i.e., 50% decline in serum IgM levels) to rituximab versus those patients who expressed phenylalanine in a homozygous pattern.

Proteasome Inhibitors

Both bortezomib and carfilzomib have been evaluated in prospective studies in patients with WM, though the latter only in combination therapy (discussed below). In a retrospective study, ten patients with refractory or relapsed WM were treated with bortezomib administered intravenously at a dose of 1.3 mg/m^2 on days 1, 4, 8, and 11 in a 21-day cycle for a total of four cycles. Most patients had been exposed to all active agents for WM and eight patients had received three or more regimens. Six of these patients achieved a partial response which occurred at a median of 1 month. The median time to progression in the responding patients is expected to exceed 11 months. Peripheral neuropathy occurred in three patients and one patient developed severe paralytic ileus in this series [162]. In a prospective study among 27 relapsed or refractory patients who received up to eight cycles of bortezomib at 1.3 mg/m^2 on days 1, 4, 8, and 11, median serum IgM levels declined significantly from 4.7 g/dL to 2.1 g/dL [32]. The overall response rate was 85%, with 10 and 13 patients achieving a minor (<25%) and major (<50%) decrease in IgM level. Responses occurred at a median of 1.4 months. The median time to progression for all responding patients in this study was 7.9 (range: 3–21.4+) months, and the most common grade III/IV toxicities were sensory neuropathies (22.2%), leukopenia (18.5%), neutropenia (14.8%), dizziness (11.1%), and thrombocytopenia (7.4%). Sensory neuropathies resolved or improved in nearly all patients following cessation of therapy. Twenty-seven patients with both untreated (44%) and previously treated (56%) disease received bortezomib, utilizing the standard schedule until they either demonstrated progressive disease or two cycles beyond a complete response or stable disease [163]. The overall response rate was 78%, with major responses observed in 44% of patients. Sensory neuropathy occurred in 20 patients following 2–4 cycles of therapy. Among the 20 patients developing a neuropathy, 14 showed resolution or improvement 2–13 months after therapy.

Combination Therapies

Because rituximab is not myelosuppressive, its combination with chemotherapy has been explored. A regimen of rituximab, cladribine, and cyclophosphamide used in 17 previously untreated patients resulted in a partial response in 94% of WM patients, including a complete response in 18% [161]. No patient had relapsed with a median follow-up of

21 months. The combination of rituximab and fludarabine used in 43 patients of whom 32 (75%) were previously untreated led to an overall response rate of 95.3%, with 83% of patients achieving a major response (i.e., 50% reduction in disease burden) [165]. The median time to progression was 51.2 months in this series, and was longer for those patients who were previously untreated and for those achieving a very good partial remission (i.e., 90% reduction in disease) or better. Hematologic toxicity was common: grade 3 neutropenia and thrombocytopenia observed in 27 and 4 patients, respectively. Two deaths occurred in this study from pneumonia. Secondary malignancies including transformation to aggressive lymphoma and development of myelodysplasia or acute myelogenous leukemia were observed in six patients in this series. The addition of rituximab to fludarabine and cyclophosphamide has also been explored in previously treated patients, of whom four of five patients had a response [166]. In another combination study, rituximab along with pentostatin and cyclophosphamide given to 13 patients with untreated and previously treated WM or lymphoplasmacytic lymphoma resulted in a major response in 77% of patients [167]. The combination of rituximab, dexamethasone, and cyclophosphamide was used as primary therapy to treat 72 patients with WM in whom a major response was observed in 74% of patients in this study, and the 2-year progression-free survival was 67% [168]. Therapy was well tolerated, although one patient died of interstitial pneumonia.

Two studies have examined cyclophosphamide, doxorubicin, vincristine, and prednisone (CHOP) in combination with rituximab (R-CHOP). In a randomized trial involving 69 patients, most of whom had WM, the addition of rituximab to CHOP resulted in a higher overall response rate (94% versus 67%) and median time to progression (63 versus 22 months) in comparison to patients treated with CHOP alone [169]. R-CHOP was also used in 13 WM patients, 10 of whom had relapsed or refractory disease [170]. Among 13 evaluable patients, 10 patients achieved a major response (77%), including 3 complete and 7 partial remissions. Two other patients achieved a minor response. In a retrospective study of symptomatic WM patients who received either R-CHOP; rituximab, cyclophosphamide, vincristine, and prednisone (R-CVP) or cyclophosphamide, prednisone, and rituximab (R-CP) and were similar in most pretreatment variables, the overall response rates to therapy were comparable among all three treatment groups—R-CHOP (96%), R-CVP (88%), and R-CP (95%)—although there was a trend for more complete remissions among patients treated with R-CVP and R-CHOP [171]. Adverse events attributed to therapy showed a higher incidence for neutropenic fever and treatment-related neuropathy for R-CHOP and R-CVP versus R-CP. The results of this study suggest that in WM, the use of R-CP may provide analogous treatment responses to more intense cyclophosphamide-based regimens while minimizing treatment-related complications. The extended alkylator bendamustine has also been evaluated in combination with rituximab in both untreated and previously treated WM patients. A randomized study by the German STiL Group examined bendamustine plus rituximab (Benda-R) versus R-CHOP in patients with untreated, indolent B-cell lymphomas including WM [172]. Patients with WM in this study showed similar overall responses (96% versus 94%), though progression-free survival was significantly longer (69 versus 29 months) in patients who received Benda-R versus R-CHOP. Treatment was also better tolerated in patients receiving Benda-R. In the relapsed or refractory setting, an overall response rate of 83% was observed with bendamustine in combination with a CD20 monoclonal antibody [173]. The median time to progression was 13 months in this study. Prolonged myelosuppression was more common in patients who received prior nucleoside analogues.

The use of two cycles of oral cyclophosphamide along with subcutaneous cladribine to 37 patients with previously untreated WM led to a partial response in 84% of patients and the median duration of response was 36 months [164]. Fludarabine in combination with intravenous cyclophosphamide resulted in partial responses in 6 of 11 (55%) WM patients with either primary refractory disease or who had relapsed on treatment [174]. The combination of fludarabine plus cyclophosphamide was also evaluated in 49 patients, 35 of whom were previously treated. Seventy-eight percent of the patients achieved a response, and the median time to treatment failure was 27 months [175]. Hematological toxicity was frequent, and three patients died of treatment-related toxicities. Two important findings in this study were the development of acute leukemia in two patients, histologic transformation to diffuse large B-cell lymphoma in one patient, and two cases of solid malignancies (prostate and melanoma), as well as failure to mobilize stem cells in four of six patients.

The combination of bortezomib, dexamethasone, and rituximab (BDR) as primary therapy in 23 patients with WM resulted in an overall response rate of 96%, and a major response rate of 83% [176]. Maintenance therapy with BDR was used in this study. The incidence of grade 3 neuropathy was approximately 30%, and led to discontinuance of bortezomib in 60% of patients on BDR. An increased incidence of herpes zoster was also observed prompting the prophylactic use of antiviral therapy. The median progression-free survival in this study was 66 months, and resolution of treatment-related neuropathy to at least grade 1 or less was observed in most (13/16; 81%) of the patients with prolonged follow-up [177].

Alternative schedules for administration of bortezomib (i.e., once weekly at higher doses) in combination with rituximab in patients with WM have achieved overall response rates of 80–90% [178, 179]. The European Myeloma Network (EMN) recently showed that transitioning bortezomib from twice-weekly intravenous dosing during the first

cycle to weekly administration thereafter reduced grade 3 neuropathy to under 10% in patients treated with BDR [180]. Overall, treatment was well tolerated and the overall response rate was 85% that included 68% major responders. The median PFS in this study was 43 months [181]. While subcutaneous bortezomib is also used to decrease the risk of treatment-related neuropathy with bortezomib, no formal studies addressing the safety and efficacy of subcutaneous bortezomib use in WM have been reported.

Carfilzomib is a proteasome inhibitor that is associated with a low risk of treatment-related peripheral neuropathy. The combination of carfilzomib with rituximab and dexamethasone (CaRD) was evaluated in WM patients [33]. Carfilzomib was administered intravenously at 20 mg/m^2 (cycle 1), and then 36 mg/m^2 (cycles 2–6), together with dexamethasone (20 mg) on days 1, 2, 8, and 9 as part of a 21-day cycle. As part of this regimen, rituximab 375 mg/m^2 was given on days 2 and 9 every 21 days. Maintenance therapy was given 8 weeks following induction therapy with intravenous carfilzomib (36 mg/m^2) and dexamethasone (20 mg) administered on days 1 and 2 and rituximab 375 mg/m^2 on day 2 every 8 weeks for up to eight cycles. Overall response rate with this regimen was 87% with major responses observed in 68% of patients, and was not impacted by MYD88^{L265P} or CXCR4^{WHIM} mutation status. With a median follow-up of 15.4 months, 20 patients remained progression free. Grade ≥2 toxicities included asymptomatic hyperlipasemia (41.9%), reversible neutropenia (12.9%), and cardiomyopathy in one patient (3.2%) with multiple risk factors. Treatment-related neuropathy occurred in one patient (3.2%) that was grade 2. Declines in serum IgA and IgG were common, and some patients required intravenous gamma globulin therapy for recurring sinus and bronchial infections.

Novel Therapeutics

The use of ibrutinib was recently approved by the United States Food and Drug Administration and the European Medicines Agency for the treatment of symptomatic patients with WM. Ibrutinib targets BTK and HCK, both targets of ibrutinib that are transactivated by MYD88^{L265P} [42, 48]. In a multicenter study that examined the role of ibrutinib in previously treated (median two prior therapies, 40% refractory) WM patients, the overall response rate was 91% [182]. Patients on this study received 420 mg a day of ibrutinib by mouth. Post-therapy, median serum IgM levels declined from 3610 to 880 mg/dL; hemoglobin rose from 10.5 to 13.8 g/dL, and bone marrow involvement declined from 60 to 25%. Decreased or resolved adenopathy was observed in 60% of patients with extramedullary disease, and five of nine patients with IgM-related PN had symptomatic improvement. At a median of 37 months of follow-up, the median progression-free and overall survival was 68% and 90%, respectively. Major responses were absent in patients with wild-type MYD88, and slower response kinetics were observed in those patients who were both MYD88 and CXCR4 mutated. Major response rates were also lower in those patients with CXCR4 mutations (62%) versus those with wild-type CXCR4 (92%). Grade ≥2 treatment-related toxicities included neutropenia (25%) and thrombocytopenia (14%) that were more common in heavily pretreated patients; atrial fibrillation associated with a prior history of arrhythmia (5%); and bleeding associated with procedures and marine oil supplements (3%). Serum IgA and IgG levels were unchanged following treatment with ibrutinib, and treatment-related infections were infrequent. A multicenter trial also examined the activity of ibrutinib in rituximab-refractory WM patients who had a median of four prior therapies. The overall response rate in this study was 90%, with major responses observed in 71% of patients. With a median follow-up of 18 months, the median progression-free and overall survival was 86 and 97% [183]. Delays in serum IgM and hemoglobin responses were observed among MYD88-mutated patients with CXCR4 mutations, versus those who were wild-type for CXCR4. One patient with wild-type MYD88 did not respond. A clinical study of the CXCR4 antagonist ulocuplumab with ibrutinib is being initiated in symptomatic WM patients with CXCR4 mutations.

Everolimus is an oral inhibitor of the mTOR pathway that is active in WM. A multicenter study examined everolimus in 60 previously treated patients that showed an ORR of 73%, with 50% of patients attaining a major response [184]. The median progression-free survival in this study was 21 months. Grade 3 or higher related toxicities were observed in 67% of patients with cytopenias constituting the most common toxicity. Pulmonary toxicity occurred in 5% of patients, and dose reductions due to toxicity occurred in 52% of patients. A clinical trial examining the activity of everolimus in 33 previously untreated patients with WM has also been reported that included serial bone marrow biopsies in response assessment [185]. The ORR in this study was 72%, including partial or better responses in 60% of patients. Among genotyped patients, nonresponders associated with wild-type MYD88 and mutated CXCR4 status. Median time to response was 4 weeks. Discordance between serum IgM levels and bone marrow disease burden was remarkable. The median time to progression was 21 months for all patients, and 33 months for major responders. Discontinuation of everolimus led to rapid serum IgM rebound in seven patients and symptomatic hyperviscosity in two patients. Toxicity led to treatment discontinuation in 27% of patients, including 18% for pneumonitis which appeared more pronounced versus previously treated WM patients.

Maintenance Therapy

The outcome of rituximab-naïve patients who were either observed or received maintenance rituximab categorical responses was examined in a large retrospective study [186]. Categorical responses improved after induction therapy in 42% of patients who received maintenance rituximab versus 10% in patients on observation. Additionally, both progression-free (56.3 versus. 28.6 months) and overall survival (>120 versus 116 months) were longer in patients who

received maintenance rituximab. Improved progression-free survival was evident despite previous treatment status, induction with rituximab alone or in combination therapy. Best serum IgM response was also lower, and hematocrit higher in those patients who received maintenance rituximab. Among patients who received maintenance rituximab therapy, an increased number of infectious events, predominantly grade 1 or 2 sinusitis and bronchitis, were observed, along with lower serum IgA and IgG levels. A prospective study examining the role of maintenance rituximab has also been initiated by the German STiL group [187]. In this study, patients received up to six cycles of bendamustine and rituximab, and responders randomized to either observation or maintenance rituximab every 2 months for 2 years. Enrollment for this study is complete, and response outcome for maintenance rituximab therapy is awaited.

High-Dose Therapy and Stem Cell Transplantation

The European Bone Marrow Transplant Registry reported the largest experience for both autologous and allogeneic SCT in WM [188, 189]. Among 158 WM patients receiving an autologous SCT, which included primarily relapsed or refractory patients, the 5-year progression-free and overall survival rate was 39.7% and 68.5%, respectively [188]. Non-relapse mortality at 1 year was 3.8%. Chemorefractory disease and the number of prior lines of therapy at time of the autologous SCT were the most important prognostic factors for progression-free and overall survival. In the allogeneic SCT experience from the EBMT, the long-term outcome of 86 WM patients was reported [189]. A total of 86 patients received allograft by either myeloablative or reduced-intensity conditioning. The median age of patients in this series was 49 years, and 47 patients had three or more previous lines of therapy. Eight patients failed prior to autologous SCT. Fifty-nine patients (68.6%) had chemotherapy-sensitive disease at the time of allogeneic SCT. Non-relapse mortality at 3 years was 33% for patients receiving a myeloablative transplant, and 23% for those who received reduced-intensity conditioning. The overall response rate was 75.6%. The relapse rates at 3 years were 11% for myeloablative, and 25% for reduced-intensity conditioning recipients. Five-year progression-free and overall survival for WM patients who received a myeloablative allogeneic SCT were 56% and 62%, and for patients who received reduced-intensity conditioning were 49% and 64%, respectively. The occurrence of chronic graft-versus-host disease was associated with improved progression-free survival, and suggested the existence of a clinically relevant graft-versus-WM effect in this study.

Response Criteria in Waldenstrom's Macroglobulinemia

Table 33.3 summarizes the response categories and criteria for progressive disease in WM based on the most recent consensus recommendations [190]. The term "overall response" is used to characterize all responses, including minor

Table 33.3 Summary of consensus response criteria for Waldenstrom's macroglobulinemia [190]

Complete response	CR	Absence of serum monoclonal IgM protein by immunofixation
		Normal serum IgM level
		Complete resolution of extramedullary disease, i.e., lymphadenopathy/splenomegaly if present at baseline
		Morphologically normal bone marrow aspirate and trephine biopsy
Very Good Partial Response	VGPR	Monoclonal IgM protein is detectable
		90% reduction in serum IgM level from baseline, or normalization of serum IgM level
		Complete resolution of extramedullary disease, i.e., lymphadenopathy/splenomegaly if present at baseline
		No new signs or symptoms of active disease
Partial Response	PR	Monoclonal IgM protein is detectable
		≥50% but <90% reduction in serum IgM level from baseline
		Reduction in extramedullary disease, i.e., lymphadenopathy/splenomegaly if present at baseline
		No new signs or symptoms of active disease
Minor response	MR	Monoclonal IgM protein is detectable
		≥25% but <50% reduction in serum IgM level from baseline
		No new signs or symptoms of active disease
Stable disease	SD	Monoclonal IgM protein is detectable
		<25% reduction and <25% increase in serum IgM level from baseline
		No progression in extramedullary disease, i.e., lymphadenopathy/splenomegaly
		No new signs or symptoms of active disease
Progressive disease	PD	>25% increase in serum IgM level from lowest nadir (requires confirmation) and/or progression in clinical features attributable the disease

responses. "Major responses" only include partial, very good partial, and complete responses. The attainment of very good partial or complete responses is associated with improved progression-free survival [165, 176, 180, 188, 191]. Response assessments in WM rely primarily on serum IgM or IgM paraprotein levels, though complete responses require disappearance of the IgM monoclonal protein, and resolution of bone marrow and/or extramedullary WM disease [190]. An important concern with the use of IgM as a surrogate marker of disease is that it can fluctuate, independent of tumor cell killing with some agents. By way of example, rituximab can induce a flare in serum IgM levels, whereas everolimus, bortezomib, and ibrutinib can suppress IgM levels independent of tumor cell killing in some patients, a finding referred to as IgM discordance [32, 156, 158, 159, 182, 185, 192]. Moreover, with selective B-cell-depleting agents such as rituximab and alemtuzumab, residual IgM-producing plasma cells are spared and continue to persist, thus potentially skewing the relative response and assessment to treatment [193]. Soluble CD27 levels have been investigated as an alternative surrogate marker in WM given their correlation with WM disease burden, and may remain a faithful marker of disease in patients experiencing a rituximab-related IgM flare, as well as after plasmapheresis [194]. The use of quantitative allele-specific polymerase chain reaction assays to assess serial MYD88^{L265P} burden in WM patients is also under investigation [35, 37, 195].

Course and Prognosis

WM typically presents as an indolent disease. The presence of 6q deletions may have prognostic significance, but does not appear to impact overall survival [29, 196, 197]. Age is an important prognostic factor (>65 years) [198–200], but is influenced by comorbidities. Anemia that reflects both marrow involvement and serum level of the IgM monoclonal protein (because of the impact of IgM on intravascular fluid retention) has emerged as a strong adverse prognostic factor with hemoglobin levels of <9 to 12 g/dL associated with decreased survival in several series [145, 198–200]. Other cytopenias also may be significant predictors of survival, and the number of cytopenias in a given patient has been proposed as a prognostic factor [199]. Serum albumin levels have also correlated with survival in some studies in WM patients [199, 200]. Elevated serum β_2-microglobulin levels (>3–3.5 g/dL) have also shown strong prognostic correlation in WM [145, 200, 201]. Several scoring systems have been proposed based on these analyses (Table 33.4), including the WM International Prognostic Scoring System (WM IPSS) which incorporates five adverse covariates: advanced age (>65 years), hemoglobin less than or equal to 11.5 g/dL, platelet count less than or equal to 100 × 10^9/L, beta2-microglobulin more than 3 mg/L, and serum monoclonal protein concentration more than 7.0 g/dL [202]. Among

Table 33.4 Prognostic scoring systems in Waldenstrom's macroglobulinemia

Study	Adverse prognostic factors	Number of groups	Survival
Gobbi et al. [198]	Hgb <9 g/dL	0–1 prognostic factors	Median: 48 months
	Age > 70 years	2–4 prognostic factors	Median: 80 months
	Weight loss		
	Cryoglobulinemia		
Morel et al. [199]	Age ≥ 65 years	0–1 prognostic factors	5 years: 87% of patients
	Albumin <4 g/dL	2 prognostic factors	5 years: 62%
	Number of cytopenias:	3–4 prognostic factors	5 years: 25%
	Hgb <12 g/dL		
	Platelets <150 × 10^9/L		
	WBC <4 × 10^9/L		
Dhodapkar et al. [147]	β_2M ≥ 3 g/dL	β_2M < 3 mg/dL + Hgb ≥12 g/dL	5 years: 87% of patients
	Hgb <12 g/dL	β_2M < 3 mg/dL + Hgb <12 g/dL	5 years: 63%
	IgM <4 g/dL	β_2M ≥ 3 mg/dL + IgM ≥4 g/dL	5 years: 53%
		β_2M ≥ 3 mg/dL + IgM <4 g/dL	5 years: 21%
Application of international staging system criteria for myeloma to WM Dimopoulos et al. [200]	Albumin ≤3.5 g/dL	Albumin ≥3.5 g/dL + β_2M < 3.5 mg/dL	Median: NR
	β_2M ≥ 3.5 mg/L	Albumin ≤3.5 g/dL + β_2M < 3.5 or	Median: 116 months
		β_2M 3.5–5.5 mg/dL	Median: 54 months
		β_2M > 5.5 mg/dL	
International Prognostic Scoring System for WM Morel et al. [202]	Age > 65 year	0–1 prognostic factors (excluding age)	5 years: 87% of patients
	Hgb <11.5 g/dL	2 prognostic factors (or age > 65 years)	5 years: 68%
	Platelets <100 × 10^9/L	3–5 prognostic factors	5 years: 36%
	β_2M > 3 mg/L		
	IgM >7 g/dL		

β_2M β_2-microbloulin, *Hgb* hemoglobulin, *NR* not reported, *WBC* white blood cell count

537 WM patients evaluated in the development of WM IPSS, low-risk patients (27%) presented with no or one of the adverse characteristics and advanced age, intermediate-risk patients (38%) with two adverse characteristics or only advanced age, and high-risk patients (35%) with more than two adverse characteristics. Five-year survival rates for these patients were 87%, 68%, and 36%, respectively. Importantly, the WM IPSS retained its prognostic significance in subgroups defined by age, treatment with alkylating agent, and nucleoside analogues. Recent data from the Surveillance, Epidemiology, and End Results (SEER) database involving 7744 WM patients showed that the relative survival of WM patients has improved over time [203]. Patients diagnosed during 2001–2010 had higher 5-year (78% versus 67%) and 10-year (66% versus 49%) relative survival rates versus patients diagnosed during 1980–2000. A Greek study that included 345 patients with WM failed to show any overall or cause-specific survival improvement in recent years, though the study might have been underpowered to detect any expected benefit [204]. However, a Swedish study of 1555 patients diagnosed with WM between 1980 and 2005 showed that the 5-year relative survival rate improved from 57% in 1980–1985 to 78% in 2001–2005 [205].

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34 Plasma Cell Leukemia

Nisha S. Joseph and Sagar Lonial

Introduction

Plasma cell leukemia (PCL) is a rare and aggressive variant of multiple myeloma that comprises roughly 1% of all myeloma cases [1]. This unique plasma cell dyscrasia is characterized by an increased number of circulating plasma cells in the peripheral blood. It can either arise de novo, as is the case in primary plasma cell leukemia (pPCL), or alternatively as a transformed leukemic phase in an end-stage previously diagnosed case of myeloma.

PCL was first described in 1906 by Professor Gluzinski and Dr. Reichenstein at the University Hospital of Lemberg, now located in present-day western Ukraine [2]. The authors reported a case of a 47-year-old male presenting with bone pain, a palpable chest wall mass, anemia, and splenomegaly. Diagnostic workup revealed elevated urine protein and immature plasma cells on the peripheral blood smear. Plasma cells were elevated at 91% in the blood, and the patient was subsequently diagnosed with multiple myeloma and "leucaemia lymphatica plasmocellularis." He was treated with arsenic-based compounds with an initial reprieve in symptoms; however the patient eventually succumbed to his disease and passed away within 6 months. Unfortunately, despite the significant progress we have seen in multiple myeloma, the prognosis of PCL has not changed much since its initial discovery.

Though the myeloma community has experienced significant advancements and progress in outcomes with the advent of novel therapeutic agents such as bortezomib and lenalidomide, followed by high-dose therapy (HDT) and autologous stem cell transplantation (ASCT), unfortunately the same success has not yet been achieved in PCL. Historically, the use of conventional chemotherapeutics such as alkylating agents, anthracyclines, and steroids in PCL has proven ineffective. The prognosis remains poor, with even worse outcomes observed in patients with sPCL. Utilization of HSCT has improved survival, but still has not achieved lasting results. With such significant success of novel agents in MM, this area warrants further exploration in PCL. The literature in this subject area is limited and our knowledge base is mainly restricted to findings from small retrospective studies and case reports. Furthermore, these studies often do not differentiate between pPCL and sPCL or between younger and older patients, making extrapolation of data to the clinical setting difficult. In this chapter, we review the clinical presentation and diagnostic evaluation of plasma cell leukemia, review the data available to date about optimal treatment options for PCL, as well as discuss future directions in research for this challenging disease.

Epidemiology

The epidemiology of PCL is very similar to that of multiple myeloma. The median age of diagnosis is 67. Primary PCL (pPCL) is more common than secondary PCL (sPCL), accounting for 60–70% of cases. Per the SEER database that surveyed patients between the years of 1973 and 2004, 291 patients with PCL were identified out of the 49,000 multiple myeloma patient in sum making the incidence of PCL 0.6%. There were no significant differences between gender, age, or race as compared with myeloma patients. Of note, this database did not distinguish between primary and secondary PCL [3].

Clinical Presentation

The clinical presentation of PCL is also very similar to that seen in patients with myeloma and leukemia. As in myeloma, many patients present with renal insufficiency, anemia, hypercalcemia, and bone pain associated with lytic lesions;

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similarly, as seen in patients with leukemia, PCL patients often present with coagulopathy, cytopenias, and hepatosplenomegaly. Other physical exam findings can include lymphadenopathy, pleural effusions, and neurologic symptoms secondary to CNS involvement. Compared with multiple myeloma, patients presenting with pPCL are more likely to have an aggressive presentation including increased tumor burden, extramedullary disease, renal involvement, and cytopenias [3].

Diagnosis

The diagnostic criteria for PCL are not clearly defined or universally agreed upon, but the diagnostic workup for a suspected case of PCL is essentially the same for that of multiple myeloma and would include peripheral blood smear, a bone marrow aspirate and biopsy, serum and urine protein electrophoresis, free light chain assay, skeletal survey, as well as chemistry panel, complete blood count with differential, and β2-microglobulin. Additional imaging and diagnostic tests such as lumbar puncture, cross-sectional imaging, or PET-CT may also be needed depending on symptoms and potential concern for extramedullary involvement. On physical examination, particular attention should be paid to bone pain, neurologic symptoms, and pleural effusions (Table 34.1).

The diagnosis of PCL must include an absolute plasma cell count >2 × 10^9/L in addition to greater than 20% circulating plasma cells of the white blood cell differential in the peripheral blood. On review of a peripheral blood smear, detection of circulating plasma cells can often be difficult morphologically as the appearance of the plasma cell can vary depending on the stage of maturity. Commonly, flow cytometry is used to confirm the diagnosis and identify the malignant clone of plasma cells. As with myeloma cells, CD138 and CD38 are often expressed whereas CD19 and CD20 are absent, and unlike myeloma, CD56 is often not expressed [4, 5].

Table 34.1 Initial diagnostic workup for plasma cell leukemia

Complete medical history and physical exam
Peripheral blood smear
Laboratory studies
CBC with differential
Comprehensive Metabolic Panel (BUN, Cr, electrolytes, Ca, Albumin)
LDH
Quantitative immunoglobulins (IgG, IgM, IgA)
β-2 microglobulin
Serum protein electrophoresis/immunofixation
Urine protein electrophoresis/immunofixation
Serum free light chain assay
Bone marrow aspirate and biopsy
Flow cytometry
FISH (t(4;14), t(14;16), t(11;14), del13, del 17p, 1 gains)
Lumbar puncture (CNS symptoms)
Imaging (As indicated)
MRI
CT scan
PET-CT

Bone marrow aspiration with biopsy is part of the diagnostic workup for pPCL for morphology and cytogenetic analysis by FISH. In PCL, pathologic review often shows an elevated number of monoclonal plasma cells with extensive infiltration of the marrow and high proliferation index. Of note, the median plasma cell percentage is higher in pPCL than in myeloma [6]. Additionally, biologic differences are seen between the myeloma plasma cell and the plasma cell seen in PCL. In PCL, malignant cells proliferate in the bone marrow as in myeloma; however these cells have an increased capacity for release into the peripheral circulation due to different expression of adhesion markers, namely NCAM (neural cell adhesion molecule/CD56) and LFA-1 (leukocyte function-associated antigen-1). Additionally, there is different expression of chemokine receptors and other genetic abnormalities that allow even more pronounced independent growth, evasion of immune detection, and prevention of apoptosis [6]. These genetic abnormalities are also seen in myeloma cells, though they tend to accumulate throughout the progression from MGUS to symptomatic myeloma, whereas in PCL, they are present at the onset. These differences may explain the extramedullary disease seen in PCL and superior evasion from immune surveillance.

Cytogenetic testing is routinely done in plasma cell dyscrasias as part of risk stratification and more recently for staging purposes. Analysis is focused on high-risk mutations such as del(17p13), del(13q), del(1p21), and amp(1q21) as well as translocations t(11;14), t(14;16), t(4;14), and t(14;20). Heavy gene translocations and hyperdiploidy are both significant oncogenic events key in the transformation to a malignant plasma cell and are seen in PCL as well. There are no distinguishable genetic features seen in PCL as compared to myeloma, but the same high-risk mutations seen in myeloma tend to occur at higher rates. Hyperdiploidy, conversely, is only found in up to 8.8% of pPCL cases whereas it is seen in ~50% of myeloma cases. This same trend is seen in sPCL, although hyperdiploidy is seen at an even higher rate (~17%) [6–9]. IgH translocations occur at a significantly higher rate in pPCL. The most commonly seen translocation in PCL is t(11;14) which used to be thought of as a favorable prognosticator in myeloma and is now more likely to be a neutral finding; however in PCL, t(11;14) is associated with

an unfavorable prognosis. The translocations t(4;14) and t(14;16) associated with poor risk in myeloma are seen in both primary and secondary PCL but at higher frequencies [3]. In PCL, an increased incidence of monosomy 13 (86% versus 26%) and a decreased incidence of trisomy 6 and monosomy 9 are seen. Additionally, it is more common to have a complex karyotype.

The differential diagnosis should include diagnoses that would also be defined by abnormal circulating cells like leukemias/lymphomas, though this can easily be distinguished with flow cytometry. It is also imperative to exclude a reactive polyclonal plasmacytosis that can be seen with bacterial or viral infections, autoimmune disorders, and serum sickness. A polyclonal plasmacytosis can be ruled out by confirming light-chain restriction on immunofixation.

Treatment

There have only been two prospective randomized trials conducted to date and these were conducted quite recently. Most of our information regarding optimal treatment in PCL is drawn from small retrospective studies and case reports; thus we have limited evidence-based data on which to base treatment decisions. For initial cytoreduction, in younger fit patients, historically aggressive chemotherapeutic regimens such as VAD and VDT-PACE were favored followed by ASCT versus chemotherapy alone in transplant-ineligible patients [10]. In the era of novel therapeutics, efforts have focused on utilization of these agents, namely lenalidomide and bortezomib (Table 34.2). These agents have shown to provide benefit but not as pronounced as in myeloma. Nevertheless, utilization of immunomodulatory agents and proteasome inhibitors has become standard of care in PCL.

Lenalidomide

The first prospective trial evaluating the optimal initial treatment in pPCL was conducted by Musto et al. and specifically studied the efficacy of lenalidomide in combination with low-dose dexamethasone in newly diagnosed pPCL. The study selected newly diagnosed pPCL patients with a performance status of 0–2, who then received lenalidomide 25 mg daily for 21 days with oral dexamethasone 40 mg given on days 1, 8, 15, and 22 on a 28-day cycle. After completion of four cycles, transplant-ineligible patients continued on with four additional cycles followed by maintenance lenalidomide at 10 mg daily on days 1–21 of a 28-day cycle until evidence of relapsed disease. Transplant-eligible patients proceeded to ASCT following four cycles of lenalidomide and dexamethasone. Patients who did not respond or progressed on initial treatment were taken off study. On intention-to-treat analysis, the overall response rate was 73.9%, and at a median follow-up of 34 months, median PFS and median overall survival were 14 and 28 months, respectively. Additionally, in evaluating the role of ASCT in treatment, PFS and OS were 27 months and not reached, respectively, in the cohort who underwent ASCT in comparison to PFS and OS of 2 and 12 months, respectively, for those patients who were transplant ineligible. Important conclusions from this study include demonstration of lenalidomide and dexamethasone as a valid frontline therapy in pPCL, as well as the important role consolidative ASCT may play as part of the larger treatment plan [11].

Bortezomib

There have been several studies that supported bortezomib-containing regimens followed by auto-SCT. Bortezomib has been shown to quickly reduce tumor volume and reverse

Table 34.2 Induction regimens for plasma cell leukemia

Author, year	*N*	Regimen	Median f/u	Best response			PFS (m)	OS (m)	ORR
				PR	VGPR	CR			
Musto, 2014	23	Rd	34 months	8 (34.7%)	6 (26.1%)	3 (13%)	14 months	28 months	73.90%
Royer, 2016	40	PAD versus VCD	28.7 months	9 (23%)	10 (26%)	4 (10%)	15.1 months	36.3 months	69%
D'Arena, 2012	29	BBR	24 months	12 (41%)	3 (10%)	8 (28%)			79%
Pagano, 2011	73	Vel ± Thal		18 (25%)		22 (30%)		12.6 months	
Katodritou, 2014	42	BBR	51 months	69%	27.50%			13 months	55%
Musto, 2007	12	BBR	21 months	5 (42%)	4 (33%)	2 (17%)	8 months	12 months	92%

PAD pegylated doxorubicin and oral dexamethasone, *VCD* bortezomib, oral cyclophosphamide, and oral dexamethasone, *BBR* bortezomib-based regimens, *Vel* bortezomib, *Thal* thalidomide

end-organ damage, namely renal dysfunction. It has also been shown to overcome the negative implication of high-risk mutations such as del17p and t(4;14) [6].

The first prospective trial to date investigating the utilization of bortezomib-based regimens (BBR) in PCL was a prospective phase two clinical trial conducted by the IFM in 2016. The aim of the study was to evaluate the efficacy of induction regimens combining both standard chemotherapy with bortezomib in conjunction with HDT and autologous stem cell transplant followed by immunomodulatory agent and/or proteasome inhibitor maintenance versus a second allogeneic transplant [12]. Forty patients were enrolled and age 70 or younger and were treated with four alternating cycles of VAD and VCD followed by ASCT. Younger, fit patients then underwent a consolidative reduced-intensity allogeneic transplant, and the remainder of patients underwent a second ASCT followed by maintenance with RVD (lenalidomide, bortezomib, and dexamethasone) for 1 year. At a median follow-up of 28.7 months, the median PFS was 15.1 months and median overall survival was 36.3 months. The overall response rate to bortezomib-containing induction was 69%. This was the first prospective trial in PCL that demonstrated that bortezomib-containing induction regimens followed by transplant led to better response rates and improved PFS.

There have been several retrospective analyses published regarding use and efficacy of bortezomib-based regimens in PCL. The largest multicenter retrospective study comes from the Italian GIMEMA working group that focused on 29 patients with pPCL who received bortezomib in varying combination regimens with other therapeutics such as dexamethasone, thalidomide, doxorubicin, melphalan, prednisone, vincristine, or cyclophosphamide. The overall response rate was ORR 79%, with 38% of subjects achieving a VGPR or better. At a median follow-up time of 24 months, 55% of these patients were living and three-quarters of these patients were in remission. The most lasting results were seen in those who had undergone HSCT following induction. Additionally, improvement in renal function was seen in 10 of the 11 patients who had presented initially with renal failure, highlighting the benefit of bortezomib particularly in those with renal dysfunction at diagnosis [13].

Another multicenter retrospective study analyzed 128 patients with plasma cell leukemia, 73 of which were classified as primary plasma cell leukemia, from January 2000 to December 2008. In this group of patients who were treated with either alkylators, anthracycline-based regimens, or bortezomib/thalidomide used as additional or single agents, it was concluded that those patients receiving thalidomide or bortezomib as first-line therapy experienced and increased duration of response by 79% [14].

Katodritou and colleagues conducted another retrospective study with the purpose of examining the efficacy of bortezomib-based regimens in 42 PCL patients, 25 with pPCL, and 17 with sPCL. BBR were given to 29 of the patients, and 6 of the 25 patients with pPCL underwent ASCT. Response rates, being defined as a PR or better, were higher in patients treated with BBR (69% versus 30.8% in those that were treated with other regimens). The ORR of pPCL treated with BBR was 88.9%, which was the highest ORR seen in the varying cohorts. With a median follow-up of 51 months, median overall survival of the BBR group was 13 versus 2 months. Between the pPCL and sPCL, median overall survival differed by 18 and 7 months. Of note, improved response rates were seen regardless of ASCT and showed treatment of PCL with BBR induces better response rates [15].

Finally, another retrospective study done by the Italian group examined patients with either primary or secondary PCL who were treated with BBR either alone or in combination with other therapies. Twelve patients were included, three of whom received bortezomib as frontline treatment, and the remaining nine patients received bortezomib after 1–4 prior lines of treatment. Important findings included an overall response rate of 92% with 50% of patients achieving VGPR or better. The median PFS and median overall survival after bortezomib were 8 and 12 months, respectively [16].

The role of immunomodulatory agents and proteasome inhibitors in PCL is promising and needs to be further explored. Additionally, combinations of these agents, which have only been studied in small patient samples, have potential and call for further study as well.

Role of Transplant

In 1983, Dr. Mcelwain and Dr. Powell in the United Kingdom first described the use of melphalan 140 mg/m^2 in a patient with pPCL, and impressively, the patient lived for 30 months after therapy [17]. HDT followed by ASCT has become the standard of care in multiple myeloma, but its role is not as well defined in PCL. Prospective randomized trials to date of HCT have excluded PCL patients. There are some case reports that have noted improved outcomes and long-term responses with ASCT/allo-SCT. The following are some of the more significant studies published regarding the role of HCT in PCL, which suggest that HCT have a role in PCL treatment; however it becomes difficult to draw definitive conclusions given the lack of randomization and no comparison of allogeneic versus autologous transplantation.

The largest retrospective study to date reviewing the role of HCT in PCL was conducted by the European Group for

Blood and Marrow Transplantation. The study identified 272 pPCL patients and compared outcomes following autologous stem cell transplant with over 20,000 myeloma patients between the years of 1980 and 2006 [18]. The median PFS following transplant of PCL versus myeloma patients was 14.3 months versus 27.4 months, and the medial overall survival was 25.7 months versus 62.3 months, respectively. Significantly improved outcomes were seen in myeloma patients, and there was higher transplant-related mortality seen in the pPCL group as well.

The Center for International Blood and Marrow Transplant Research (CIBMTR) led another large retrospective study published in 2012. Of 147 patients with pPCL, 97 underwent autologous transplant and 50 underwent allogeneic HCT. Significant findings include the following: a median PFS of 34%, median overall survival at 3 years was 64% versus 39%, and relapse rate at 3 years was 61% versus 38% in the autologous group versus allogeneic group, respectively. Though the relapse rate was significantly lower with allogeneic transplant, transplant-related mortality was significantly higher (41% versus 5% in the autologous transplant cohort). Given better OS with autologous transplant in light of increased mortality associated with allogeneic transplant, this study supported use of ASCT with improved outcomes and decreased toxicities [19].

Another multicenter retrospective analysis performed by Pagano et al. looked at 73 patients with pPCL. In those patients who had undergone HSCT, they experienced longer overall survival and duration of response at 83.1 and 25.8 months, respectively, in contrast to 9.1 and 7.3 months seen in non-transplanted patients. Impressively, this analysis revealed an increase in mean overall survival by 69% and duration of response by 88%; however though this study supported use of HSCT, it unfortunately did not differ between autologous and allogeneic transplantation.

Prognosis

In general, the prognosis for PCL remains poor. These dismal outcomes are likely attributable to the increased incidence of the same high-risk mutations seen in myeloma, namely del17p, t(14;16), and t(4;14). As in myeloma, these mutations are noted to be of high risk due to the more aggressive nature and higher proliferative rate of these malignant cells. Mean overall survival is less than 1 year, and even worse in those patients with secondary PCL in the context of relapsed/refractory multiple myeloma. There has been modest improvement in outcomes with incorporation of ASCT in conjunction with novel agents. This is best demonstrated by a registry study including 445 patients which shows improved median survival from decade to decade, particularly when entering the early 2000s which encompasses the time period when novel agents were introduced in myeloma. Median survival through the decades trended as follows: from 1973 to 1995 median overall survival was 5 months, from 1996 to 2000 median overall survival was 6 months, from 2001 to 2005 median overall survival was 4 months, and then notably from 2006 to 2009 median overall survival improved to 12 months [20]. The most significant improvement has been seen in older patients, with early mortality decreasing from rate of 26 to 15% since the 1970s. Additional prognostic value can be drawn from patient's response to treatment. In those whose disease does not respond to initial therapy, prognosis is generally only a few months. Response to induction is defined as at least 50% reduction in circulating plasma cells within the first 10 days of treatment or complete clearance within 1 month [21].

Future Directions

There continues to be significant advances and study of novel therapeutics for multiple myeloma, and this provides the opportunity for continued study in the PCL subset. There are numerous ongoing clinical trials, some of which include study of efficacy of newer proteasome inhibitors (carfilzomib and ixazomib), peptide vaccines, second-generation immunomodulatory agent pomalidomide in combination with ixazomib, and the kinesin spindle inhibitor ARRY-520, among several others (Table 34.3). Future areas for exploration also include investigating monoclonal antibodies elotuzumab and daratumumab and utility of CAR T-cells. Of note, a new phase two prospective trial for pPCL being conducted by the European Myeloma Network is currently under way. The study looks at the use of carfilzomib in combination with lenalidomide and dexamethasone (KRD), followed by autologous transplant, then a second autologous transplant versus a non-myeloablative allogeneic transplant, and finally maintenance therapy with carfilzomib and lenalidomide. The findings of this study are eagerly anticipated and will provide further insight into the efficacy of these newer agents.

Table 34.3 Future directions for therapy in PCL: current therapeutic options being actively studied in clinical trials for PCL as single-agent and/or combination regimens

Carfilzomib (second-generation proteasome inhibitor)
Ixazomib (third-generation proteasome inhibitor)
Pomalidomide (second-generation immunomodulatory agent)
Peptide vaccines
CAR T-Cells
Venetoclax (BCL-2 inhibitor)
ARRY-520 (kinesin spindle inhibitor)
Elotuzumab, daratumumab (monoclonal antibodies)

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35 Prognosis of Myeloma/Genetics of Myeloma

Sébastien Robiou-Du-Pont, Jill Corre, and Hervé Avet-Loiseau

Multiple myeloma (MM) is a very heterogeneous disease. This heterogeneity is observed at all the levels: clinical, biological, and molecular. This heterogeneity translates in extremely variable length of survival, from a few weeks to more than 15 years, and even cure. MM is probably the cancer for which the highest number of prognostic parameters have been described. These prognostic factors can be divided in three parts: related to the patient's conditions, related to disease burden, and related to the tumor clone itself.

Prognostic Parameters

Patient's Conditions

The first parameter is age. As for many diseases, patients diagnosed at an earlier age display a better outcome. Several factors contribute to this survival variability. Older patients more frequently present comorbidities (cardiac, renal, pulmonary, hepatic) which could prevent the optimal delivery of therapy. Another major parameter is related to the treatment schema. It has been shown that high-dose melphalan, with autologous stem cell rescue, improved at least the progression free survival (PFS), and even overall survival (OS). However, this intensive approach is not feasible in the oldest population. Historically, the cutoff has been set at 65 years of age. With the improvement of patients' condition, it is now currently set at 70 years. All these parameters translate in a shorter OS in elderly patients, in the 5–6 years range.

Tumor Burden

As in many cancers, the clinical stage is associated with outcome. In MM, this fact has been shown by Durie and Salmon [1]. Their staging system correlated with the tumor burden, even if this system is currently abandoned for prognosis assessment. The tumor burden is reflected, at least partially, by the serum β2-microglobulin level [2]. This protein is expressed at the surface of the malignant plasma cells, and is shed by proteases, and released in the serum. The outcome is linearly correlated with the β2-microglobulin levels, the highest levels being associated with the shortest survivals. This serum level is also correlated with renal function, since the protein is eliminated via the kidney. Whether the level of β2-microglobulin is prognostic in case of renal failure is still a matter of debate. β2-Microglobulin level is the basis of the International Staging System (ISS), in association with the serum albumin level (Table 35.1) [3]. This system divides the patients in three groups, with very significant differences in both PFS and OS, independently of age and treatment approach. Other parameters have been linked to the tumor burden, and especially the bone marrow involvement. Even though the mechanisms of anemia and thrombocytopenia are probably very complex in MM, it has been clearly shown that both factors were associated with a shorter OS [4].

Table 35.1 International staging system

ISS 1	β2m < 3.5 mg/L and Albumin ≥35 g/L
ISS 2	All other cases
ISS 3	β2m > 5.5 mg/L

β2m = β2-microglobulin

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Tumor Clone

Several characteristics of the tumor clone are associated with outcome. First, extra-medullary localizations are clearly associated with shorter survival. This is particularly obvious in the case of primary plasma cell leukemias, with a median OS of less than two years [5]. The causes of these extra-medullary developments are poorly understood, and might be related to the default of bone marrow homing factors expression. The second parameter is the tumor proliferation. It can be evaluated by the plasma cell labeling index (assessed by flow cytometry), or by conventional cytogenetics [6, 7]. High proliferation correlates with shorter survival. But the most important prognostic factor is certainly genetics, meaning the chromosomal/molecular abnormalities observed in the tumor plasma cells.

Genetics

Myeloma is characterized by many chromosomal changes [8]. Even though conventional karyotypes are often normal (because of the low proliferative index of the tumor plasma cells), analyses based on SNP array did show that the large majority of patients present chromosomal changes, with usually complex molecular karyotypes. Multiple myeloma can be divided in two groups based on ploidy, i.e., hyperdiploidy and non-hyperdiploidy. Hyperdiploidy is observed in at least 50% of the patients, with a nonrandom gain of specific chromosomes, i.e., chromosomes 3, 5, 7, 9, 11, 15, 19, and 21 [9]. The causes of these nonrandom gains are currently totally unknown. Non-hyperdiploidy is characterized by usual more complex karyotypes, displaying many structural abnormalities (partial gains and losses), with a specific enrichment of 14q32 translocations (60–70% of the cases). These 14q32 translocations systematically involve the *IGH* gene at 14q32, with several chromosomal partners. The most frequent partners are *CCND1* at 11q13 (15–20% of the patients) [10], *MMSET* and *FGFR3* at 4p16 (12–15% of the patients) [11], and *MAF* at 16q23 (3% of the patients) [12]. These specific translocations are due to errors during the physiological class switch and hypermutation processes. What is unknown so far is the specificity of the partners.

Many other chromosomal changes have been identified, including monosomy 13, gain of 1q, and losses of the 1p, 8p, 14q, 16q, and 17p regions. At the molecular level, frequent rearrangements of the *MYC* locus have been described. Recent exome sequencing studies revealed another level of heterogeneity [13–16]. No specific gene mutation has been observed. The most frequently mutated genes were *KRAS* (~20%), *NRAS* (~15%), *DIS3*, *TP53*, *BRAF*, and many others. Compared with other hematopoietic tumors, and especially with leukemias, MM present a high number of mutations. In contrast, if compared with solid tumors, the mutation load is low.

Prognostic Impact of Chromosomal Abnormalities

Many of the chromosomal changes observed in the tumor plasma cells are associated with specific outcomes (Table 35.2). Very few "good risk" abnormalities have been described, in contrast to "high-risk" changes. Most of these prognostic values have been described with old treatment strategies, and have to be reevaluated in the context of novel therapies.

High-Risk Features

The first chromosomal abnormality associated with a shorter PFS and OS has been del(13q14) [17]. Actually, this chromosomal loss is mainly due to chromosome 13 monosomies. Further studies did show that monosomy 13 does not present an intrinsic prognostic value [4]. The shorter PFS and OS observed in old studies were in fact related to the frequent association of monosomy 13 with the specific t(4;14) and del(17p) (80% of those cases).

The loss of part of the chromosome 17 short arm has been associated with very dismal outcome [18]. The minimal target region is currently unknown, even though all the studies did focus on the 17p13 region, and more specifically on the *TP53* gene. The *TP53* gene is a tumor suppressor gene, and per se, has to be mutated on the second allele to display its oncogenic properties. Several studies did address this issue, and none of them did show a systematic mutation on the remaining allele [14, 19], in contrast to what has been observed in chronic lymphoid leukemia or diffuse large B-cell lymphoma. In the 30–50% of the cases presenting a *TP53* mutation, *TP53* is probably the target gene. In the 50–70% remaining cases, other gene(s) are probably the cause of the prognostic impact of del(17p) losses. A recent study in a mouse model of leukemia/lymphoma did suggest that the loss of several genes in the *TP53* vicinity is associated with tumorigenesis [20]. This paradigm has to be dem-

Table 35.2 Prognostic value of cytogenetic abnormalities

t(4;14)	High Risk
Del(17p)	High Risk
1q gain	High Risk
Del(1p32)	High Risk
Trisomy 3	Good Risk
Trisomy 5	Good Risk
Trisomy 21	High Risk

onstrated in MM. However, all the cases of del(17p) (7–10% of the patients) are associated with a poor outcome, independently of *TP53* mutations. This poor outcome is not related to lower response to therapy, but to a shorter PFS. Recent clinical studies in relapse patients with novel proteasome inhibitors such as carfilzomib and ixazomib did show that this prognostic impact might be overcome, at least partially [21, 22]. This important finding has to be confirmed in the frontline setting, but may lead to a new paradigm in the therapeutic strategy for these specific patients. One hot topic is the relationship between the prognostic impact and the size of clone harboring the deletion. It has been suggested that the deletion is impacting the survival only if present in the major subclone [4]. This issue is currently evaluated.

The second important prognostic parameter is the translocation t(4;14). The target gene on chromosome 14 is *IGH*. This gene is targeted in many B-cell malignancies, but in contrast to the other translocations, the t(4;14) is peculiar since it disrupts two genes on chromosome 4: *FGFR3* and *MMSET* [11]. The *FGFR3* gene is displaced on chromosome 14, leading to its overexpression in a classical mechanism. In contrast, the *MMSET* gene remains on chromosome 4, and is upregulated through a novel fusion gene, *Eμ-MMSET*. The most important event is probably this latter one, for at least two reasons: (1) in 1/3 of the patients presenting the t(4;14), *FGFR3* is lost through an unbalanced translocation [23], and (2) the prognostic impact of the translocation is the same, independently of the translocation configuration [24]. The *MMSET* gene is a methyl-transferase, and its upregulation leads to a chromatin configuration modification, that may modify the expression of many target genes. Observed in 12–15% of the patients, the prognostic value of t(4;14) has been clearly shown in the context of old drugs [4]. Since the availability of proteasome inhibitors such as bortezomib, its prognostic impact is more questionable. Several studies did show that bortezomib may partially overcome this impact [25], and this fact has been recently confirmed in studies using second generation proteasome inhibitors such as carfilzomib and ixazomib [21, 22].

Other factors have been shown to negatively impact the prognosis. Extra-copies of the chromosome 1 long arm (1q gains) are observed in about 1/3 of the patients, and have been shown to be associated with a shorter survival [26]. However, the prognostic value of 1q gains seems to be lower than del(17p) or t(4;14). Since in the large majority of the cases the whole 1q is gained, it is difficult to find a single target gene which could drive the prognosis. The chromosome 1 does also drive the prognosis through deletions of the short arm (del(1p)). Several regions can be involved, but the most important seems to be the 1p32 region, targeting the *FAF1* and/or *CDKN2C* genes [27]. These del(1p32) are observed in 7–8% of the patients, and are associated with a dismal outcome.

Table 35.3 Revised-international staging system

R-ISS 1	ISS 1 *and* no high-risk cytogenetics *and* LDH = Normal
R-ISS 2	All other cases
R-ISS 3	ISS 3 *and* [high-risk cytogenetics *or* LDH > Normal]

High-risk cytogenetics = del(17p), or t(4;14), or t(14;16)

Good/Standard Risk Features

Some studies did suggest that hyperdiploidy may be associated with a better outcome. However, these studies were based on conventional cytogenetics, and so the results are restricted to patients presenting a proliferative disease. Two studies did address the issue of the impact of trisomies in the high-risk patients, with totally opposite results [28, 29]. A third study based on SNP array did show that only some specific trisomies (chromosome 3 and 5) improve the outcome of high-risk features. In contrast, another trisomy (chromosome 21) worsens the prognosis [30].

In conclusion, cytogenetics is probably the most important prognostic parameter in MM. But all the abnormalities should be analyzed more globally, some high-risk changes could become standard risk if combined with some trisomies. A first attempt has been proposed by IMWG with the R-ISS (Revised-International Staging System), which combines ISS with chromosomal abnormalities (Table 35.3) [31]. In the future, global analyses including ISS and multiparametric genetic analyses may contribute to define high risk more precisely, but also good and standard risk groups.

Prognostic Impact of Gene Expression Profiling

Besides the chromosomal changes, MM is also characterized by abnormalities in the gene expression profiles (GEP) . In 2006, the Arkansas group proposed a MM classification based on similarities of GEP [32]. This classification is mostly driven by the chromosomal changes, i.e., 14q32 translocations and hyperdiploidy. Two other subgroups, corresponding to “proliferation” and “bone disease” were identified. This molecular classification has been partially confirmed by a study by the HOVON group [33]. The “low bone disease” group was not confirmed. In contrast, three other groups were identified: one group enriched by “myeloid” genes (that could be related to plasma cell sorting problems), one group characterized by overexpression of cancer testis antigen genes, and finally a group defined by overexpression of positive regulators of the NFκB pathway. However, these classifications, based on gene expression, were not translated into prognostic subgroups. Different studies based on GEP did identify a high-risk group [33–35]. Of note, these different prognostic models, based on variable

number of genes, do not share any common gene. Whether these discrepancies are due to different algorithms to identify the high-risk signatures, or to differences in the treatments used in the training cohort is still unresolved.

Prognostic Impact of Next Generation Sequencing

Next generation sequencing (NGS) has been used to analyze the different mutations present in the tumor plasma cells. At least four studies based on exome sequencing have been published, with a total of more than 700 patients analyzed [13–15, 36]. So far, preliminary results are rather disappointing, without the identification of significant group of patients who would present a good or poor prognosis. All these studies describe subclonality, but none of them did show that this process can be useful in either prognostic assessment, or adaptation of treatment strategies. Whether the number of mutations per patient is prognostic is still unresolved.

Conclusions

The assessment of prognosis in patients with MM is mandatory at diagnosis. The most important variables are age, ISS, and cytogenetic changes, summarized in the R-ISS. In the future, the development of multiparametric systems will probably enable to improve this assessment.

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Immunoglobulin Light Chain Amyloidosis (AL)

36

Morie A. Gertz, Francis K. Buadi, Taimur Sher, and Angela Dispenzieri

History

"The term 'lardaceous change' has … come more into use chiefly through the instrumentality of the Vienna School… The term 'lardaceous changes' … has but very little to do with these tumors, and rather refers to things, upon which the old writers … who are better connoisseurs in bacon than our friends in Vienna, would hardly have bestowed such a name [1]… The appearance of such organs … are said to look like bacon, bears … a much greater resemblance to wax and I have, therefore, now for a long time … made use of the term waxy change… These structures … are the simple action of iodine … assume just as blue a color as vegetable start…"

In this publication by Rudolph Virchow [2], he decides that amyloid must be made of starch. The iodine reaction turning amyloid deposits blue is a throwback to high school chemistry when iodine will stain the open face of a potato blue, documenting it as starch. At the same time, Prof. Virchow directly insults his chief competitor of the day, Rokitansky, who had previously written about lardaceous changes because of the greasy texture the liver of an amyloid patient would have and implies that the Viennese School is incapable of distinguishing amyloid from bacon, reflecting the intense competitive nature of two leaders during the age of medical discovery.

The term "amyloid" was first used in 1838 by Schleiden to describe a normal constituent of plants. Virchow, in 1858, gave a lecture entitled, "Amyloid Degeneration." Virchow's conclusion that amyloid substance was starch continues today, where amyloid means amyl-like or starch-like. In 1859, Friedreich, Nikolau, and Kekule indicated that the waxy spleen described by Virchow did not contain starch and that the deposits were derived from protein.

Budd analyzed the liver of a patient with amyloidosis and found it was not lardaceous. Wilks described a 52-year-old patient that had lardaceous change that was unrelated to any secondary cause and may have been the first description of AL. Schmiedeberg, in 1920, indicated that amyloid was composed of amino acids and strongly resembled the composition of serum globulin. In 1922, Bennhold first used Congo red as a specific stain. Five years later, Divry and Florkin reported green birefringence under polarized light when amyloid-laden brain from an Alzheimer's patient was stained with Congo red. In 1931, Magnus-Levy postulated that Bence-Jones proteins were a precursor of the amyloid substance and noted that there was a relationship between amyloid, Bence-Jones protein, and multiple myeloma. Cohen and Calkins published in 1959 that, under electron microscopy, all forms of amyloid were fibrils of indefinite length but a constant width of 9.5 nm [3]. Apitz claimed that amyloid in tissues was analogous to the excretion of light chain proteins by the kidneys and coined the term "paraprotein" to describe monoclonal immunoglobulins. Isobe and Osserman, in 1974, published that Bence-Jones proteins had a direct role in the pathogenesis of AL [4]. In 1968, Eanes and Glenner reported by X-ray diffraction [5] that amyloid proteins formed an alternate three-dimensional configuration of a beta-pleated sheet [6] unlike the normal alpha-helical configuration of proteins. Amyloid proteins are highly resistant to solvents, and the first amyloid extraction relied on this insolubility by repeated centrifugations in saline where the supernatant, containing all the soluble compounds, would be

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discarded, and the residual pellet contained all the amyloid and could be suspended in distilled water. Amyloid was first purified in 1968 [7]. The first sequence of an immunoglobulin light chain amyloid was reported in 1970 as the N-terminal fragment of the immunoglobulin light chain.

Introduction

Amyloid is a vague term that describes a group of disorders that have only one thing in common, and that is the deposition of protein fibrils composed of protofibrils. The clinical presentation depends on the organ involved and the protein subunit structure of the amyloid protein. These heterogenous disorders are classed together because they share common tinctorial properties of an amorphous eosinophilic deposit when stained with hematoxylin and eosin, and distinct binding to the cotton-wool dye, Congo red, in the sine qua non of green birefringence when viewed under polarized light [8]. Congo red is an imperfect stain insofar as there is a measurable false-positive rate either due to trapping of dye in thick sections or the misinterpretation of white birefringence as green and points out the importance of having positive and negative controls whenever amyloid is diagnosed.

The immunoglobulin fragments that compose AL amyloidosis are thermodynamically unstable and will demonstrate misfolding into the β-pleated sheet configuration [9]. It is possible to synthetically create amyloid fibrils by pepsin digestion of monoclonal immunoglobulin light chains.

Injection into mice of purified immunoglobulin light chains from the urine of patients with multiple myeloma does not produce any pathologic deposits. However, when light chains extracted from the urine of patients with amyloidosis are injected into mice, amyloid deposits do develop, reflecting the importance of [10] the amino acid structure of the immunoglobulin light chain in predisposing to misfolding into an amyloid configuration. Amyloid associated with the λ6 subgroup of light chains is virtually always associated with amyloidosis, and nearly 60% of patients with light chain amyloidosis have a λ light chain compared to only one-third of patients that have multiple myeloma [11].

As part of the standard evaluation of light chain amyloidosis, a bone marrow is routinely performed. Patients that fulfill criteria for multiple myeloma with >10% plasma cells or have myeloma-associated CRAB criteria (hypercalcemia, cast nephropathy, anemia due to marrow infiltration or bone lesions) have a shortened survival when compared with patients with light chain amyloidosis and <10% plasma cells, and this has important therapeutic implications with regard to systemic chemotherapy [12]. Renal insufficiency in amyloidosis is not due to cast formation in the tubule but due to progressive destruction of the glomerular basement membrane and loss of glomeruli associated with long-standing proteinuria that results in tubular atrophy [13].

Amyloidosis does not appear to be neoplastic, although it is clonal. Monitoring of the bone marrow over time in a patient with amyloidosis does not show the proliferative characteristics that myeloma patients have where, left untreated, the bone marrow plasma cell percentage rises over time [14]. In light chain amyloidosis, the disorder is far more static. Moreover, although patients with light chain amyloidosis have a high frequency of t(11;14), high-risk features such as seen in multiple myeloma such as -17p, t(4;14), or t(14;16) are lacking [15]. If multiple myeloma is not present at the time of diagnosis, it will subsequently develop in <1% of patients. Even in those patients that have a high proportion of plasma cells in the bone marrow, the cause of death is generally amyloid-related organ failure and not the typical problems associated with end-stage myeloma such as pancytopenia and infection [16, 17].

Amyloidosis is thought to occur in approximately eight patients per million per year and is approximately one-fifth as common as multiple myeloma [18]. Although recent estimates are not available, since the current incidence of multiple myeloma in the United States is 24,000 new patients per year, it is reasonable to speculate that the number of new patients with light chain amyloidosis is approximately 5000 annually. Translocations of the immunoglobulin heavy chain locus located on chromosome 14, band 14q32, have been reported in 55% of patients with light chain amyloidosis. Cyclin D1 overexpression accounts for 76% of all IgH translocations [19].

Symptoms and Signs of Amyloidosis

The most common symptoms associated with amyloidosis are weight loss, fatigue, edema, dyspnea on exertion, and paresthesias [20]. Unfortunately, these common symptoms of AL are nonspecific and are generally not helpful in determining when the diagnosis should be suspected and when to launch an investigation. Extreme weight loss usually results in a futile search for metastatic malignancy [21]. Fatigue, which can be related to early cardiac or renal involvement, can be quite subtle since the restrictive cardiomyopathy is usually associated with a normal ejection fraction and a cardiac etiology may be overlooked. Reduced filling, seen in the heart during diastole, will result in a decline in systolic blood pressure, but it is often difficult to associate this with the presence of an infiltrative cardiomyopathy [22]. Typically, patients with cardiac amyloid have normal coronary arteries; and not infrequently, coronary angiography is performed, and the patient's symptoms are interpreted as being noncardiac in origin [23].

Lightheadedness and orthostatic syncope occurs in a significant proportion of patients [24]. Patients that have significant proteinuria and hypoalbuminemia lose oncotic effect, and serum will, therefore, transude from the intravascular space into the extravascular, extracellular space. This results in contraction in the intravascular volume and can lead to

orthostatic hypotension. Diuretics used to manage the edema that patients with hypoalbuminemia have will frequently result in further hypotension and reduced renal blood flow and resultant rise in serum creatinine level. When these patients undergo echocardiography, it is common to see thickening of the walls, which is often interpreted as hypertrophy, which is then considered a reflection of untreated hypertension or workload hypertrophy, since valvular insufficiency is often seen on echocardiography [25].

Occasional patients will have autonomic failure as the cause of their orthostatic hypotension. This is generally associated with peripheral neuropathy and other signs of autonomic dysfunction, such as upper intestinal dysmotility with pseudo-obstruction and vomiting or lower intestinal dysmotility with alternating obstipation and intractable diarrhea [26]. Since the symptoms associated with light chain amyloidosis are often vague, clinicians may rely on the signs of amyloidosis to lead to a diagnosis. Unfortunately, the signs, which are highly specific, are quite insensitive. The classic pinch or periorbital purpura (Fig. 36.1) seen with amyloidosis is seen in <20% of patients [27]. Clues are that the purpura tends to stop at the nipple line; can be periorbital or petechiae on the eyelids; and can be seen on the webbing of the neck, malar regions, and upper chest. These purpura have been misinterpreted as being senile purpura in an elderly population. Hepatomegaly is present in only 10% of patients and is rarely over 5 cm below the right costal margin. Splenomegaly and splenic rupture are rarely seen [28].

Macroglossia is the most specific finding of amyloidosis (Fig. 36.2). Patients can be misdiagnosed as tongue cancer, acromegaly, and hypothyroidism [29]. Tongue enlargement with submandibular indentations, due to pressure on the lower row of teeth, is quite specific for immunoglobulin light chain amyloidosis and, virtually, is never seen in AA or TTR amyloidosis. Tongue enlargement, however, is seen in only approximately 10% of patients and is very easy to overlook unless massive and interferes with the patient's ability to swallow or concomitant sleep apnea when supine. Tongue enlargement is associated with bilateral enlargement of the submandibular salivary glands. Dry mouth can be seen and can be misdiagnosed as Sjögren syndrome [30].

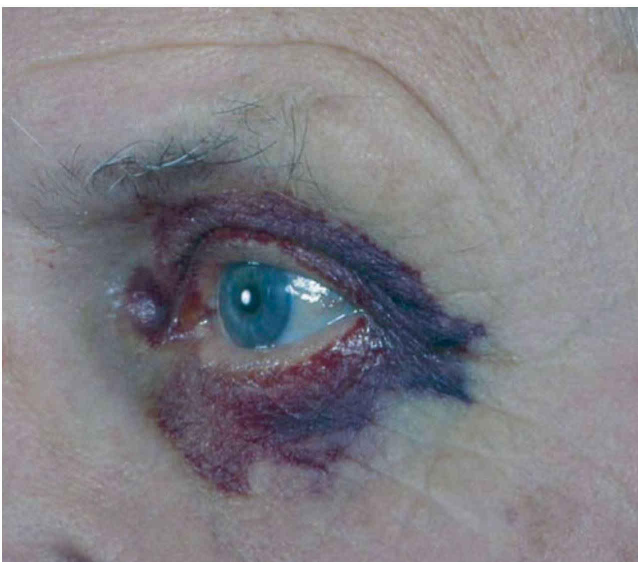

Fig. 36.1 Periorbital purpura in amyloidosis

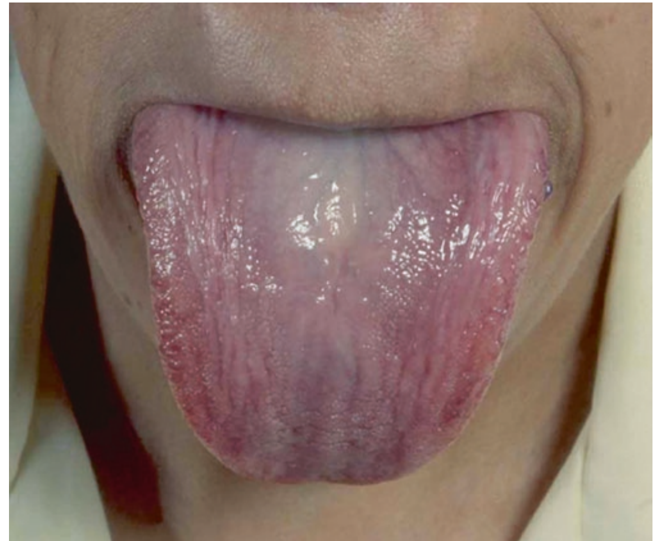

Fig. 36.2 Enlarged tongue in an AL amyloidosis patient

Occasional patients have diffuse small vessel amyloidosis that can cause calf, jaw [31], or buttock claudication with exertion and is difficult to distinguish from pseudoclaudication related to spinal stenosis. A rare patient will have coronary arteriolar amyloid deposits that will result in exertional angina [32]. A very rare finding is periarticular amyloid deposition leading to the shoulder-pad sign (Fig. 36.3), which represents pseudohypertrophy of the shoulder joint. As a consequence, if one waits for the signs of amyloidosis to develop, the majority of patients will be overlooked [33].

Diagnosis of Amyloidosis

The diagnosis of amyloidosis is best suspected when patients fulfill one of the five following criteria [34] (Fig. 36.4):

1. Diastolic heart failure, heart failure with preserved ejection fraction, or infiltrative cardiomyopathy
2. Nephrotic range proteinuria in a nondiabetic
3. Unexplained hepatomegaly with alkaline phosphatase elevation with no history of malignancy
4. A progressive demyelinating peripheral neuropathy with paresthesias that is generally painless
5. A patient presenting to the hematologist with "atypical" multiple myeloma where fatigue and edema are the

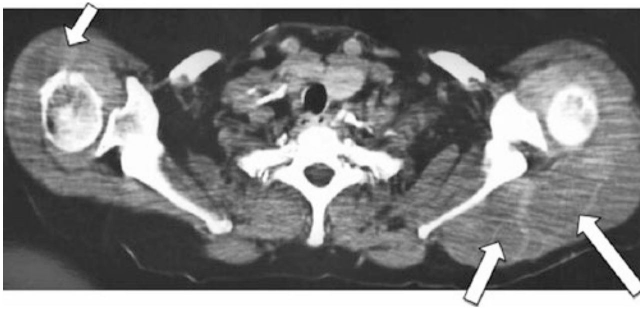

Fig. 36.3 CT scan of the shoulder showing periarticular infiltration (shoulder pad sign)

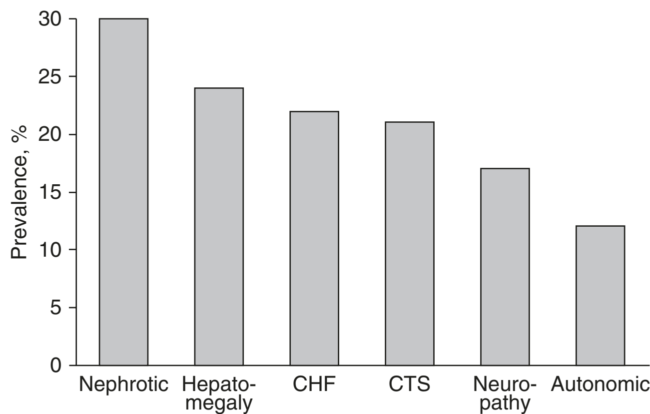

Fig. 36.4 Prevalence of clinical syndromes in AL

presenting symptoms rather than the classical CRAB criteria of anemia, renal insufficiency, or bone disease

In patients that present with any of these symptoms, the first screening should be immunofixation of the serum and an immunoglobulin free light chain assay [35]. Nearly all patients with immunoglobulin light chain amyloidosis will have an abnormal involved free light chain or the presence of a monoclonal protein on serum immunofixation. A positive result would be a powerful clue to the origin of the patient's symptoms and is quite sensitive with only 1% of amyloid patients failing to have a monoclonal protein [36]. The test is specific since only 3% of the adult population will have an incidental monoclonal gammopathy of undetermined significance. Using sensitive techniques, whether immunohistochemistry or flow cytometry of the bone marrow, a clonal population of plasma cells can be demonstrated [37]. All patients with a monoclonal gammopathy of undetermined significance that have any symptoms of dyspnea or fatigue should have the urine screened and should be screened with cardiac biomarkers to exclude the possibility of an infiltrative cardiomyopathy [38].

A clonal population of plasma cells is observed in virtually all patients with AL, even when the marrow percentage of plasma cells is 1–2% [39]. The amyloid deposits of light chain amyloidosis are derived, usually, from a fragment of the immunoglobulin light chain variable region. The source of this light chain (which averages approximately 12 kDa or half the molecular weight of a normal intact immunoglobulin light chain) is the clonal plasma cell population. An immunoglobulin free light chain assay is important for screening any patients with dyspnea, neuropathy, and proteinuria. Serum immunofixation will fail to detect a monoclonal light chain in nearly a quarter of patients. The light chain levels are low in AL, and a high percentage of the light chain passes through the glomerular basement membrane into the urine, and there is no discernible monoclonal peak in the serum [40]. The immunoglobulin free light chain assay is ten times more sensitive than serum immunofixation, capable of detecting levels as low as 2 mg/dL; where serum protein electrophoresis detects peaks of 200 mg/dL, and the estimated sensitivity of immunofixation is 20 mg/dL [41].

The plasma cells in patients with light chain amyloidosis are λ in nearly 70% of patients. If a patient with known amyloidosis does not have a monoclonal protein in the serum, polyclonal bone marrow plasma cells, and normal levels of immunoglobulin free light chain, the amyloidosis should be considered either localized or, if systemic, it should be considered an inherited or acquired form of non-AL until proven otherwise [42].

All forms of amyloid contain approximately 15% glycoprotein by weight, consisting of amyloid P component. Mass spectroscopic analysis identifies P component in all amyloid deposits [43]. The function of amyloid P component remains unknown, but no human has ever been described lacking amyloid P component, suggesting it performs a vital function. Outside of the United States, imaging with I123-labeled amyloid P component can be used to identify amyloid deposits in vivo [44]. Patients that have high amyloid burdens have shorter survival. P component is also a potential target of antibody therapy as will be described in the treatment section [45].

Serialized imaging with P component has been used to assess response and progression to therapeutic interventions [46]. P component scanning is incapable of distinguishing among the various forms of amyloid deposits. The heart cannot be imaged with the P component scan, and kidneys are not well seen. The correlation between imaging findings and the extent of organ dysfunction assessed biochemically is not good. However, the diagnostic sensitivity of SAP scintigraphy for AL amyloidosis is 90% [47].

Biopsy Proof of Amyloidosis

At the time of diagnosis, amyloidosis is widespread, and extensive involvement of microvessels is characteristic. As a consequence, it is possible to biopsy nearly any site to obtain a diagnosis. Although biopsy of an affected organ (such as heart, kidney, liver, and nerve) will yield a diagnosis, the widespread nature of the disorder allows one to establish a histologic diagnosis noninvasively. Biopsy of the skin [48], subcutaneous fat, bone marrow [49], and gingiva [50] are highly sensitive and easily accessible (Fig. 36.5). Fine needle aspiration of the fat will yield a diagnosis in 75% of patients [51], but a trial comparing subcutaneous fat aspiration with surgical biopsy demonstrated a higher yield with surgical biopsy [52]. Biopsy of these less-invasive sites reduces the risk of bleeding that has been reported with liver and kidney biopsies. Endoscopic biopsy of the stomach, jejunum, and colon will also yield a diagnosis in nearly 95% of patients, usually localized to submucosal blood vessels [53]. Endoscopy can be performed then as an outpatient, and the risk of GI hemorrhage is quite small. Labial salivary gland biopsy has been reported for its ability to diagnose amyloidosis noninvasively [54].

The specificity of a subcutaneous fat aspirate is 99%, and the false-positive rate is 1%. Concordance between pathologists is 95%, and the tissue is suitable for mass spectroscopic analysis of the amyloid deposits [55]. At our center, trained nurses perform both the subcutaneous fat aspiration and the bone marrow biopsy as a single procedure that allows two samples to be submitted for analysis and an overall sensitivity to detect amyloidosis of 85%. A bone marrow is required to estimate the percentage of plasma cells, and it is convenient to do both procedures at a single setting. Caution is required when interpreting the Congo red stain because of the risk of false-positives [56]. Rectal biopsy specimens [57] with amyloid have been misinterpreted as collagenous colitis. As a screening technique in our laboratories, the myocardial biopsies are stained with sulfated Alcian blue; and in our peripheral nerve laboratory, crystal violet screening is used subsequently confirmed with Congo red [58].

Identifying the Type of Amyloid

Once amyloid has been recognized by a pathologist on tissue section, classification of the type of amyloid is required. Immunohistochemical staining with commercial antisera has been promoted as a highly sensitive technique. There are major drawbacks with the use of antibody-mediated techniques to identify amyloid deposits [59] (Fig. 36.6). Since the immunoglobulin protein in light chain amyloidosis is only a fragment of the immunoglobulin light chain, constant portions may be deleted [60]. Most commercial antisera identify light chains by binding to the constant region, and with the deletion of constant immunoglobulin fragment the epitope cannot be recognized [61]. Moreover, one characteristic of the light chains in amyloid is misfolding of the light chain, which can result in suppression of the epitopes that commercial antisera recognize buried within the misfolded protein [62]. As a consequence, particularly for immunoglobulin light chain amyloidosis, immunohistochemistry lacks sensitivity. It has been repeatedly demonstrated that immunohistochemistry is an excellent technique for the recognition of both TTR and AA amyloidosis. Unfortunately, there are over a dozen recognized forms of amyloidosis, which include

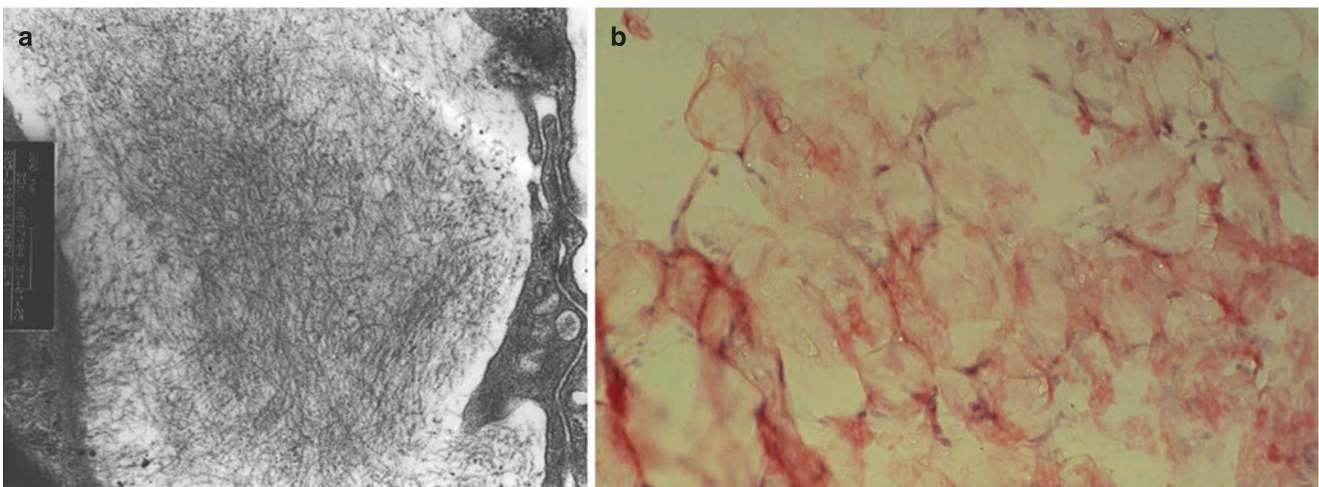

Fig. 36.5 (**a**) Electronic micrograph of amyloid fibrils (published from the prior book chapter). (**b**) Congo red stained fat aspiration

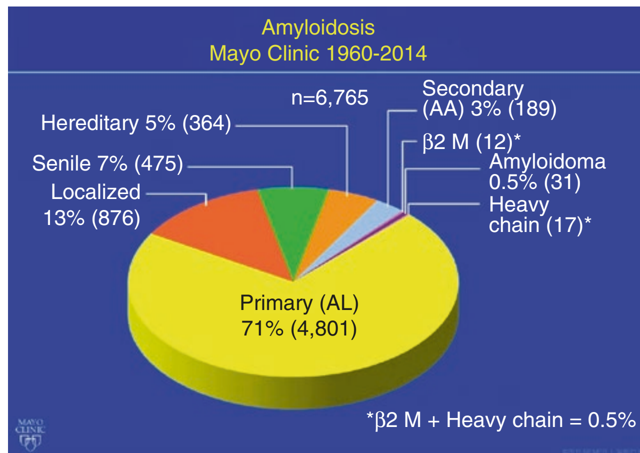

Fig. 36.6 Amyloid type seen at Mayo Clinic

Table 36.1 Types of amyloid identified in tissues

Amyloid subtype	Percentage
AL (light chain)	61.7
ATTR (familial or wild type)	24.5
AA (secondary)	3.7
ALECT-2 (renal)	3.6
A Ins (localized insulin)	1.1
Keratin (cutaneous)	0.9
A Apo 1 (inherited)	0.7
AH (heavy chain)	0.7
A Fib (hereditary renal)	0.6

apolipoprotein, lysozyme, insulin, gelsolin, etc. [63]. Most laboratories are not adequately configured to perform immunohistochemical studies for all forms of amyloidosis. Currently, the gold standard for the identification of amyloid deposits is laser capture microdissection mass spectroscopic analysis of the amyloid deposits [64]. Sequencing of the proteome and subsequent comparison with a library of protein sequences is virtually 100% sensitive in identifying the primary structure of the amyloid protein subunit [65] (Table 36.1). LCMS is also capable of identifying other proteins such as apolipoprotein E, serum amyloid P, and vitronectin, which are common in amyloid deposits and validate that the deposit is indeed amyloid [43]. Using this technique, we have demonstrated that 38% of amyloid deposits are not of immunoglobulin light chain origin, and an increasing number of new proteins have been identified, including Alect2, keratin, A-fibrinogen, and A-atrial natriuretic factor [66].

One of the great dangers in clinical practice reflects the high prevalence of monoclonal gammopathies in the serum of the elderly, ranging from 3% prevalence at age 70 years to 5% at age 90 years. This is the same population that has a high incidence of wild-type TTR cardiac amyloidosis. Therefore, the finding of classic amyloidosis in the heart of an elderly male that has a monoclonal protein does not necessarily indicate cardiac AL amyloidosis. A tissue specimen is required in order to exclude wild-type TTR amyloidosis, and mass spectroscopy is well suited to make this distinction [8, 67].

Distinguishing Localized and Systemic Amyloidosis

Even when mass spectroscopic analysis demonstrates immunoglobulin light chain as the primary protein subunit of the amyloid deposits, it does not prove that it is systemic. There are a number of localized forms of AL amyloidosis that can involve skin, bladder, larynx, and gastrointestinal tract and not be evidence of a systemic syndrome [68–70]. Supportive evidence in these instances is the absence of a monoclonal protein in serum and urine, a normal immunoglobulin free light chain assay, and failure to find a clonal population of plasma cells in the bone marrow. At Mayo Clinic, nearly 16% of patients seen with amyloidosis have localized disease. Amyloidosis involving the renal pelvis [71], ureter, bladder [72], and urethra can present with hematuria, unilateral renal obstruction, mimicking colic, or imaging findings that would be suggestive of ureteral and bladder carcinoma, respectively. Patients with tracheobronchial amyloidosis will present with hoarseness, poor vocal cord movement, and occasional stridor. Patients with genitourinary and tracheobronchial amyloid do not benefit from systemic therapy and need to be treated locally.

Pulmonary nodules that have all the characteristics of a solitary malignancy can represent localized nodular amyloidosis [73]. These deposits are AL in origin, are unassociated with a systemic light chain disorder, and do not require therapy.

Cutaneous deposits of amyloid are usually found to represent keratin [74] deposits and are 1% of all amyloid proteins analyzed at Mayo Clinic. These lesions cause pruritus but are not at risk of developing a systemic form of amyloidosis. Localized amyloidosis has also been described in the conjunctiva [75]. Management is by an oculoplastic surgeon. Conjunctival amyloid can often be confused with conjunctival lymphoma. Carpal tunnel syndrome is seen in 15% of patients with light chain amyloidosis, but there is a separate syndrome of localized carpal tunnel amyloidosis, which has been demonstrated to be composed of TTR [76]. Men who have wild-type TTR cardiac amyloidosis have a history of carpal tunnel syndrome in nearly 50% [58]. Trace amounts of amyloid can be found in the cartilage of the hip and the knee and represent incidental findings. There is also a form of localized atrial amyloidosis that is composed of atrial natriuretic factor and contributes to atrial arrhythmias but is not associated with systemic cardiac amyloidosis with ventricular dysfunction [77]. Whenever amyloidosis is detected, exclusion of localized amyloid that does not require chemotherapy is essential. Amyloid deposits found in the cardiac ventricle, kidney, and liver virtually always represent systemic AL amyloidosis even if only a single organ is involved.

Systemic Forms of Amyloidosis that Are Not Immunoglobulin Light Chain in Origin

The clinical presentation of systemic non-AL amyloidosis involving nerve, kidney, and heart are not distinguishable from light chain amyloidosis. Patients with renal amyloidosis can be composed of light chains, Alect2, fibrinogen-Aα, or be a manifestation of AA amyloid [78]. In all instances, there is proteinuria or renal insufficiency. Most patients with light chain, AA and fibrinogen amyloid present with nephrotic range proteinuria. Patients with Alect2 actually have modest degrees of amyloid deposition with significant elevations in serum creatinine, and histology shows preferential deposition in the tubular and interstitial regions of the kidney [79]. Apolipoprotein amyloid, also an inherited form of amyloid preferentially involving the kidney, presents with creatinine elevation and only modest degrees of proteinuria in the range of 1 g/24 h [63].

The differential diagnosis of systemic cardiac amyloid is AL amyloid; mutant TTR amyloid, formerly known as familial amyloid cardiomyopathy; and wild-type TTR cardiac amyloid, formerly known as senile cardiac amyloid or senile systemic amyloid [80]. Mutant TTR amyloidosis has echocardiographic features similar to AL amyloidosis but is 90% men, usually over the age of 60 years, with a high incidence of associated carpal tunnel syndrome [81]. The extent of amyloid infiltration seen on echocardiography is usually greater than that in AL, suggesting that the fibrils of TTR amyloid are less toxic to the myocardium [82]. It is not unusual to see an interventricular septal thickness of 20 mm in TTR amyloidosis, where this would be unusual in AL amyloidosis. Increased recognition has led to a surge in the diagnosis of wild-type TTR amyloidosis as the willingness of cardiologists to biopsy the heart in older individuals is increasing and as new investigational therapies become available. Wild-type TTR amyloidosis also has a much better prognosis than light chain amyloidosis [83], with survivals of 5–7 years, compared with AL amyloidosis of 1–2 years. Recently, it has been demonstrated that radionuclide scanning with either technetium pyrophosphate [84] or technetium DPD [85] is quite specific for TTR amyloidosis. The finding of a compatible echocardiogram with restrictive cardiomyopathy and poor filling with a positive technetium imaging scan (Fig. 36.7 PYP or DPD) is diagnostic of TTR amyloidosis and is a useful distinguishing feature.

Amyloidosis and the Heart

The heart is the most important organ to be involved with amyloidosis and drives the prognosis. Amyloid is deposited extracellularly and results in the thickened and noncompliant left ventricle that is seen on echocardiography and magnetic resonance imaging [86]. Because systolic function is pre-

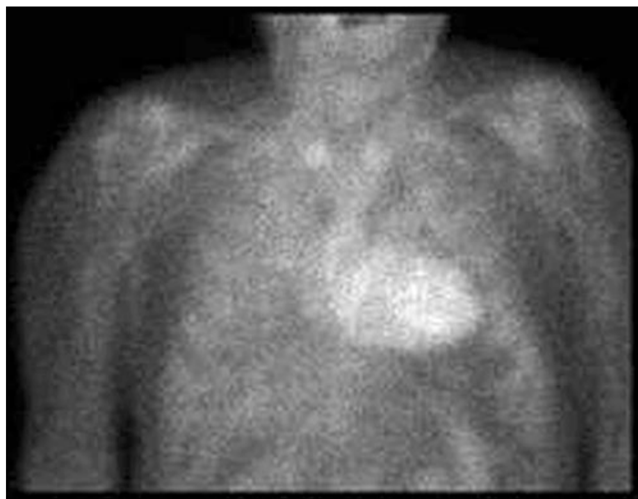

Fig. 36.7 PYP scan of TTR amyloidosis

served until late in the course and cardiomegaly is not seen on a chest radiograph, it is easy to misattribute the thickening to a more common systemic illness such as hypertension or valvular disease. Coronary angiography is typically normal. The median septal thickness for patients with immunoglobulin light chain amyloidosis is 14 mm (normal ≤11) [87]. Thickening of the septum has also been misattributed to asymmetric septal hypertrophy. The finding of a monoclonal protein in a patient with “hypertrophic cardiomyopathy” or “hypertensive cardiomyopathy” should lead to an evaluation for amyloidosis [88]. Doppler studies on echocardiography are quite useful in demonstrating the restriction to inflow and the rapid rise in diastolic filling pressures characteristic of the restrictive physiology of amyloidosis [22]. Wall thickness and fractional shortening are predictive of survival in this disease [89]. The recent introduction of strain echocardiography allows measurement of a rate at which the myocardial wall shortens; normal is −18% or less (−20% normal). Amyloid is characterized by rises (−15%, −12%, etc.) and is shown to predict survival in patients with amyloidosis [90]. Patients with amyloidosis of the heart can develop atrial thrombi, which can be a potential source of arterial embolization and stroke [91]. There is a high incidence of supraventricular rhythm disturbances that are resistant to ablation therapy [92]. There is a high incidence of sudden death [93], and nearly 30% of patients with cardiac amyloidosis die within the first 2 months of recognition [94]. There is a rare syndrome of intramural coronary arteriolar amyloidosis. These patients present with classic anginal symptoms with normal extramural coronary arteries seen after angiography [95].

A particular area of misdiagnosis is in elderly African-American men, 3% of whom carry a mutation for TTR V122I [96]. The finding of cardiac amyloidosis in an African-American male necessitates mass spectroscopic analysis of the deposits due to the high prevalence of this form of

inherited TTR amyloidosis. The echocardiographic features of these forms of amyloidosis are indistinguishable, and all are positive for Congo red, necessitating careful analysis of the amyloid protein subunit.

There is increasing recognition of wild-type TTR cardiac amyloidosis, formerly known as senile systemic amyloid and senile cardiac amyloid. These patients have the same echocardiographic features as other patients with cardiac amyloid but tend to be older, nearly 90% are men, nearly half have associated carpal tunnel syndrome, and renal involvement is conspicuously absent [97]. The severity of the heart failure is less for the extent of infiltration identified by echocardiography, and median survival is longer than that in cardiac AL. These patients do not benefit from systemic chemotherapy, and the mechanism by which a normal protein, such as TTR, preferentially deposits in the myocardium of elderly men remains unknown. In an observational study, patients with mutant TTR amyloid had a survival of 25.6 months inferior to those with wild-type TTR amyloidosis at 43 months. In a subsequent analysis of 272 patients with wild-type TTR, the mean age was 77; 89% were men and the median survival was 3.5 years. We have seen rare patients with wild-type TTR amyloid under the age of 60. So the overlap is quite substantial, and age alone cannot be used as a distinguishing feature. The clinical spectrum of wild-type TTR amyloidosis is quite broad. It is the obligation of the hematologist to ensure that these patients are not inappropriately administered cytotoxic chemotherapy [98].

The mechanism whereby immunoglobulin light chains and amyloid produce cardiac dysfunction is not well understood [99]. Light chains produce oxidative stress, cellular dysfunction, and apoptosis in adult cardiac myocytes through activation of p38 mitogen-activated protein kinase [100, 101]. The presentation of cardiac amyloidosis is heart failure with a preserved ejection fraction [58]. Again, hypertrophy, asymmetric septal hypertrophy, and hypertrophic cardiomyopathy have all been incorrectly diagnosed in patients with light chain cardiac amyloidosis. The electrocardiogram in light chain amyloidosis is neither sensitive nor specific. Low voltage is found in only 46%, a pseudo-infarct in 47%, and 16%, despite infiltration, had EKG findings that met criteria for LVH [102]. Since this is a diastolic disorder, ejection fraction remains normal until advanced disease onset. The standard for evaluating cardiac amyloidosis is the echocardiogram, showing increased wall thickness, left ventricular outflow obstruction, peak systolic tissue velocity, abnormal systolic strain, and abnormal systolic strain rate compared with patients without amyloidosis [103]. Doppler imaging is used to detect impaired left ventricular diastolic filling. In 249 consecutive patients, strain rate imaging predicted mortality and was independent of age and New York Heart Association class as well as cardiac biomarkers [104]. Not all patients with cardiac amyloid have thickening of the ventricular walls; 36% of patients in one trial had a left ventricular wall thickness of ≤12 mm with a median survival of 2 years [105].

Radionuclide imaging serum amyloid P component will bind to all forms of amyloid and will demonstrate deposits but not in the heart. Technetium 99 M derivatives, including pyrophosphate and DPD [106], are capable of binding to TTR cardiac amyloid and are a useful test to distinguish AL from ATTR amyloidosis. Technetium DPD is primarily used in Europe, and Technetium PYP [84] is primarily used in the United States. Carbon 11-PIB with PET scanning has been used to study amyloidosis affecting the heart after it was shown to be effective in diagnosing Alzheimer's plaques; this is ongoing [107].

Cardiac Magnetic Resonance Imaging

MRI is an effective technique when cardiac amyloidosis is suspected. It shows ventricular wall thickening, ventricular wall mass, and a characteristic late gadolinium enhancement [108, 109] (Fig. 36.8). The use of gadolinium in patients

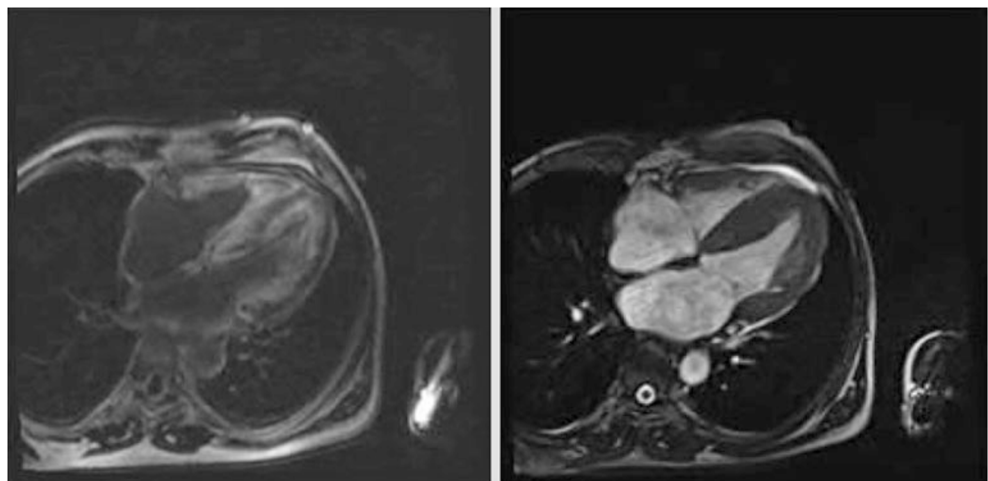

Fig. 36.8 MRI of heart showing amyloid

with renal amyloid is unwise because they are at risk of developing late systemic fibrosis [110]. Cardiac MRI can identify 91% of patients with restrictive filling patterns. Expansion of the extracellular space indicative of amyloid deposition in the heart results in circumferential late shortening of the endocardium and thickening of the myocardial wall. Diffuse transmural late gadolinium enhancement correlates with interstitial amyloid infiltration and correlates with other clinical indicators such as New York Heart Association class and left ventricular wall thickness.

Biomarkers

Although echocardiogram and cardiac magnetic resonance imaging has been a standard for diagnosis for nearly three decades, there are issues of interobserver variability and reproducibility so that serial changes of the echocardiogram over time have not been validated as being predictive of survival. Serum biomarkers that indicate myocyte injury (troponin) or left atrial stress (N-terminal propeptide of B natriuretic peptide (NT-proBNP)) are more reproducible and have been incorporated into systems designed both to stage and predict outcomes in patients with amyloidosis [16, 111].

The symptoms of cardiac amyloidosis are nonspecific. Poor diastolic filling, low left ventricular end-diastolic volume, and reduced cardiac output result in reduction in systolic blood pressure. Levels of troponin T and NT-proBNP can be used in conjunction with the immunoglobulin free light chain level to determine patient's stage and median survival and can be used to predict early [112] mortality with autologous stem cell transplantation [113]. The main cause of death in patients with all forms of light chain amyloidosis (whether hepatic, renal, or peripheral nerve) is cardiac involvement with subsequent heart failure or sudden death. In a multivariable model of over 800 patients with AL amyloidosis, the concentration of cardiac troponin T and NT-proBNP independently predicted overall survival. The cutoffs were NT-proBNP > or < 332 ng/mL and troponin T of 0.035 μg/L. By assigning one point for each, patients could have both normal, one abnormal, or both abnormal, resulting in three stages [114]. It has subsequently been demonstrated that the DFLC is prognostic as is the percentage of plasma cells in the bone marrow and whether the patient has multiple myeloma in the bone marrow or any CRAB criteria, all of which shorten the survival in patients with light chain amyloidosis [12].

The new system has four stages, where one point is assigned for a cardiac troponin T level > or < 0.025 μg/L, an NT-proBNP level ≥ 1800 ng/L, and a clonal free light chain burden >18 mg/dL. Investigators at University College London Amyloid Center have further refined stage 3 into stage 3A (BNP between 1800 and 8500) and stage 3B (all features of stage 3 with an NT-proBNP > 8500) [115]. This results in four (or five) stages. The median survival in the Mayo system for the four stages, respectively, is 94.1, 40.3, 14, and 5.6 months (Table 36.2).

Table 36.2 Amyloid staging system [112]

1 Point each for:
dFLC >18 mg/dL
cTnT >0.025 ng/mL
NT-proBNP >1800 pg/mL

Median survivals: 0 = 94.1; 1 = 40.3; 2 = 14; 3 = 5 months

Renal Amyloidosis

In a patient with nephrotic range proteinuria, measurement of immunoglobulin free light chains, if abnormal, will rapidly narrow the possibilities into myeloma cast nephropathy, Randall light chain deposition disease, light chain amyloidosis, and cryoglobulinemia [116]. In most instances, the finding of light chains in the serum and in the urine will obviate the need for renal biopsy since biopsy of the fat and bone marrow will usually demonstrate the presence of amyloid without having to proceed with the risks of renal biopsy. It is important that all patients with proteinuria have immunofixation performed of the serum, urine, and a free light chain assay. The kidneys are affected in nearly 40% of patients with AL amyloidosis; and for nondiabetic adults with nephrotic range proteinuria, amyloid is 12% of renal biopsy specimens. If one looks at all renal biopsy specimens that are performed, amyloid represents 2.8%. A recent system looking at the initial urinary protein and the serum creatinine at diagnosis is useful for predicting the risk of dialysis (see Table 36.3) [117]. The amount of urinary protein has no impact on overall survival, which is driven by the presence or absence of cardiac involvement.

Two-thirds of AL patients with immunoglobulin light chain amyloidosis have detectable light chains in the urine or in the serum. Monoclonal light chain proteins are more common when the urinary protein loss is high. Symptomatically, the massive proteinuria leads to hypoalbuminemia, and the decline in oncotic pressure results in progressive edema, requiring diuretics. However, diuretics often will further

Table 36.3 Renal staging system [117]

Proteinuria >5 g/24 h
eGFR <50 mL/min
Risk at dialysis at 3 years:
Both favorable: 0–4%
Both unfavorable: 60–85%
1 Unfavorable: 30%

decrease intravascular volume, which can result in significant hypotension and decline in renal vascular blood flow, with resultant rise in the serum creatinine level. Approximately one-third of our patients have an antecedent history of a marked change in the level of serum cholesterol, and the rapid rise in cholesterol is a manifestation of the evolving nephrotic syndrome. In our experience, the median time from diagnosis of nephrotic syndrome to dialysis was 14 months [118]. After initiation of dialysis, the median survival time was 8 months, with patients usually succumbing to cardiac involvement. There does not appear to be any reported differences between hemodialysis and peritoneal dialysis in terms of outcome [119]. Effective chemotherapy will reduce urinary protein loss and slow the progression to end-stage renal disease and ultimately improve survival [120]. Concomitant cardiac amyloidosis makes dialysis quite difficult to complete because of hypotension with each dialysis run. There is no correlation between the amount of amyloid in the glomerulus and the extent of urinary protein loss. The urinary sediment is rather benign with fat or fatty acid crystals, but the sediment is not inflammatory or nephritic. Occasionally, amyloid patients will present with adult Fanconi syndrome with crystalline deposits in the proximal tubules and resultant urinary loss of uric acid, phosphorus, amino acids, and potassium. Immunotactoid glomerulopathy can be confused with light chain amyloidosis, but the majority of patients with immunotactoid fibrils in the kidney do not have an associated plasma cell dyscrasia [43]. The fibrils seen by electron microscopy in immunotactoid glomerulopathy do not stain with Congo red. Randall light chain deposition disease is very hard to distinguish from light chain amyloidosis. However, light chain deposition disease rarely involves liver, heart, and nerves [121, 122].

Therapy

Supportive Care Treatment for Amyloidosis

For both cardiac and renal amyloidosis, the mainstay is diuretic therapy. Diuretic therapy can be complicated because so many patients have orthostatic hypotension and intravascular volume contraction due to their hypoalbuminemia. Diuretic therapy can raise the creatinine level and lower the systolic blood pressure. Our cardiologists appear to have a preference for torsemide over furosemide as having better bioavailability for patients; and for those patients in whom loop diuretics fail to control edema, the addition of Metolazone in doses from 2.5 mg QOD to 5 mg BID can be beneficial but can be complicated with relatively severe hypokalemia. Patients that develop orthostatic syncope are often treated with fludrocortisone or midodrine. The latter is complicated by severe supine hypertension.

Cardiac arrhythmias are a common problem in patients with amyloidosis; and clearly, if syncope can be documented to be related to severe bradycardia or supraventricular tachyarrhythmia that results in poor filling in between beats, a permanent pacemaker can be placed [123]. There is no evidence that afterload reduction with ACE or ARBS is of any benefit for patients with cardiac amyloidosis, and it has been our observation that patients tolerate these medications poorly and symptomatically feel worse. Moreover, because of poor diastolic filling, many of these patients require a rapid heart rate to maintain their stroke volume. As a result, artificially lowering a rapid heart rate with beta blockers can result in a decline in cardiac output and symptomatic deterioration in patients. Our cardiologists tend to avoid the use of beta blockers in amyloidosis patients [51].

There is data to suggest that an implantable defibrillator can be of benefit in patients for whom ventricular fibrillation has been diagnosed or those with recurrent syncope that cannot be attributed to orthostatic hypotension [93, 124] and is likely to be related to high-grade cardiac arrhythmias [125]. Digoxin is relatively contraindicated in the management of amyloid heart disease because there has been a previously reported high prevalence of sudden cardiac death. However, there are instances where it can be used to increase AV nodal blockade and reduce the heart rate in patients with rapid ventricular response. Calcium channel blockers have been reported to precipitate congestive heart failure and are best avoided in the management of these patients.

A small number of patients with cardiac amyloidosis have been reported with a left ventricular assist device as both destination therapy and as a bridge to transplantation. The numbers are too small to actually determine the appropriate role of a left ventricular assist device [126].

Cardiac transplantation has been used at multiple centers for amyloid, but major problems exist [127]. Systemic chemotherapy is clearly required. Without chemotherapy, one can expect recurrence of amyloid into the allografted heart [128]. Virtually all reported outcomes suggest that patients with heart transplant for amyloid do not do as well as patients with cardiomyopathy. As long as organ availability remains an issue, the question arises for every amyloid patient that gets a heart, which patient did not get a heart? Different centers have different philosophies about chemotherapy either before or after cardiac transplant. On one side, systemic chemotherapy is given before cardiac transplant in order to ensure chemotherapy sensitivity and the ability to control light chains. The downside of this is patients with advanced cardiac failure who are candidates for heart transplants do not tolerate significant amounts of systemic chemotherapy, and a real test of the ability to control the plasma cell dyscrasia cannot be accomplished. Unfortunately, delaying chemotherapy until after a heart transplant runs the risk that patients will be chemotherapy nonresponsive after they have received an allografted

heart, and this raises major challenges in trying to prevent recurrence. The Mayo Clinic standard has been to give patients an allografted heart followed by an autologous stem cell transplant [129]; and if there is persistent disease, use bortezomib-based chemotherapy as part of consolidation [130]. Cardiac transplantation does not bring up any major technical problems compared with patients receiving an allograft for cardiomyopathy, but outcomes will not be favorable without treatment of the underlying plasma cell dyscrasia [131]. Between May 1992 and November 2012, 178 adult patients referred for cardiac transplant for amyloidosis were evaluated; 78 did not complete evaluation, 94 completed evaluation, and 38 were considered ineligible primarily because of extracardiac amyloidosis, which we consider exclusion criteria for transplant; 56 were listed for transplant and 31 received transplant, 22 of whom had AL amyloidosis. The overall survival of the patients was a median of approximately 4 years with 20% alive at 10 years. Survival following heart transplant of amyloid patients was 81.6% and at 5 years, 48%. This compares with non-amyloid patients who have a 1- and 5-year survival of 93.4 and 83.6, respectively [132]. Chemotherapy and/or stem cell transplant following a heart transplant may significantly improve survival and needs ongoing study. The role of cardiac transplant in AL amyloidosis remains uncertain. Other groups have performed cardiac transplantation reported through the UNOS database, and 69 patients were reported in aggregate with 5 operative deaths and 29 late deaths and 9 deaths attributed to amyloid-related complications. The 5 patients had sequential cardiac and stem cell transplantation; 2 died of progressive amyloid 33 and 90 months after heart transplant; 1 patient actually had an allogeneic bone marrow transplant. In the United Kingdom, 24 patients with amyloid heart disease were reported with cardiac transplantation, 17AL [133]. One-, two-, and five-year survival rate was 86%, 86%, and 64%, respectively. Progression of amyloid systemically contributed to increased mortality.

Supportive care for renal amyloidosis includes hemodialysis for those patients who go onto end-stage renal disease, but their outcomes are inferior related to the high proportion of patients that have concomitant cardiac amyloidosis. Renal transplantation has also been performed for these patients [134]. The majority at Mayo Clinic receive a living related kidney because of the long wait for a cadaver donor [135]. These patients also require systemic chemotherapy because amyloid deposits in the transplanted kidney are well reported [136]. In a report of 45 patients who underwent kidney transplantation, the 3-year survival rate was 51%, with recurrent amyloid in the transplanted kidney detected in four. The estimated recurrence rate of amyloid at 1 year after transplant was 20%. No specific predictors of recurrence were identified. Sequential living donor renal transplant and autologous stem cell transplant is feasible. We have reported eight such patients with six long-term survivors.

Chemotherapy Treatment for Light Chain Amyloidosis

The primary therapy currently available for the treatment of light chain amyloidosis is plasma cell-directed therapy. Therapies are capable of destroying the underlying plasma cell clone responsible for the synthesis of the immunoglobulin light chains. These chains ultimately become resistant to catabolic breakdown and subsequently misfold into the amyloid configuration. Alkylator and steroid therapy remains one of the viable options for the treatment of amyloidosis, but none of the available regimens have been subjected to rigorous phase III randomized clinical trials [87]. The first use of alkylating agents dates back to 1972. The use of high-dose, alkylating-agent chemotherapy followed by autologous stem cell transplantation dates back over 20 years [137]. The ultimate goal of therapy is not reduction of the M component the way it is in multiple myeloma where response depth is a prediction of improved survival. In light chain amyloidosis, the goal of plasma cell-directed chemotherapy is to eventually invoke an organ response with improved cardiac, renal, or hepatic function. Because response to chemotherapy is time-dependent and there is a lag time between a hematologic response and an organ response, there are a number of patients that will succumb due to advanced organ failure before sufficient time elapses to allow for reducing the burden of toxic circulating light chains. The first alkylator combination was melphalan and prednisone, and two phase III trials have been performed using colchicine as the comparator arm that showed benefit [138]. Unfortunately, the majority of patients failed to achieve a hematologic response; and as in multiple myeloma, the doublet of melphalan and prednisone has been relegated to historical interest only. Early patients treated with melphalan and prednisone have clear-cut organ regression and improvement with SAP scintigraphy demonstration of a reduction in amyloid deposits.

The Mayo Clinic experience with melphalan and prednisone reported an objective hematologic response rate of 18%. Moreover, patients presenting with a serum creatinine >3 mg/dL were nonresponders and destined to go on to dialysis-dependent renal failure. The best responses were seen in renal amyloid nephrotic syndrome, but patients with amyloid cardiomyopathy can definitely respond; and melphalan and prednisone can be dose-adjusted so that it can be administered to virtually any patient of any age with any degree of cardiac or renal dysfunction [139]. Although only a few patients actually respond to melphalan, when they do, median survival is as long as 89 months. For melphalan-based therapy, it can be a challenge to know when to abandon the therapy since organ responses can be delayed. A trial of bortezomib-based chemotherapy and melphalan-dexamethasone failed to demonstrate a survival advantage

for the bortezomib arm [140]. Even in the era of melphalan and prednisone, 10-year survivors were regularly seen, almost all responders to alkylating-agent chemotherapy. Predictors of poor outcome, as in all trials of amyloid, include heart failure, age, serum creatinine, and percentage bone marrow plasma cells. When melphalan-prednisone was compared to colchicine, median survival in the melphalan-prednisone arm was 17 months compared to 8.5 months. These short survivals are a reflection both of the late diagnosis commonly seen with melphalan treatment of amyloidosis as well as the relatively low frequency with which response was achieved. Melphalan, particularly with prolonged exposure, has a significant risk of MDS or ANLL. In a series of 153 closely followed patients exposed only to melphalan, ten ultimately developed therapy-related MDS or ANLL [141]; eight of the ten showed classic cytogenetic features, which include hypodiploidy, deletion of chromosome 5, or deletion of chromosome 7. The actuarial risk for developing myelodysplasia at 42 months was 21%, reflecting the small proportion of long-term survivors. Today, melphalan is rarely given for longer than 12 months; and with the multiple alternatives available, reexposure to melphalan is uncommon, and rates of MDS that we are currently seeing are approximately 1%.

Approximately 10 years ago, melphalan-prednisone was supplanted with melphalan and high-dose dexamethasone [142]. Most patients were selected on the basis of stem cell transplant ineligibility. In an original trial of 46 patients, a hematologic response was seen in 67%, with an organ response in 33%, where hematologic response was the most powerful predictor of organ response [143]. This oral regimen had only a 4% all-cause mortality at day-100 and resulted in resolution of cardiac failure in 6 of 32 patients, with a median time to response of 4.5 months. In a follow-up trial of 93 patients, complete hematologic responses were seen in 24%, and improvement in amyloid-related organ failure was seen in 45%, with a 2-year event-free survival of 52%. In this trial, heart failure and β2 microglobulin levels predicted adverse outcome and durable reversal of amyloid-related organ dysfunction. Since patients on chemotherapy trials of amyloidosis are usually cared for at a trial center, there is inherent referral bias with regard to the outcomes, which is not reflective of the real-world experience.

Stem Cell Transplantation for the Management of Amyloidosis

Since the first report of five patients receiving high-dose therapy with stem cell transplant for the treatment of patients with amyloidosis, hundreds of articles have been published, demonstrating the benefits of stem cell transplantation in this disorder [144]. In multiple myeloma, patients tend to have unhealthy bone marrow but tend to have preserved vital organ function. Unfortunately, in patients with amyloidosis, comorbidities associated with cardiac failure, hypoalbuminemia, renal insufficiency, and chronic hepatic dysfunction lead to major complications after the administration of high-dose melphalan. The visceral organ dysfunction, which is always part of amyloidosis, has resulted in early mortality rates reported as high as 10% [145]. A mortality level this high was previously acceptable when the number of standard-dose chemotherapeutic alternatives was small but would not be considered an acceptable mortality rate in an era when multiple alternatives to transplantation exist. Most centers collect stem cells in amyloidosis without mobilizing chemotherapy. Although the majority of patients can be collected with growth factor alone, there has been an increasing tendency to give plerixafor [146] to enhance peripheral blood stem cell collection [147]. Patients with amyloid have a tendency to retain a good deal of fluid during filgrastim mobilization of peripheral blood stem cells [148, 149]. Although many centers incorporate plerixafor on a routine basis, our policy is to measure the peripheral blood CD34 on day-5; and if it is <10, we would begin plerixafor that evening and initiate collections the following morning. We dose plerixafor at 0.24 mg/kg when the creatinine clearance is >50; and for patients with a creatinine clearance <50, we reduce the dose to 0.16 mg/kg, and we never exceed a maximum dose of 24 mg. Even using this regimen, we find a high proportion of patients that still retain fluid; and in up to 20%, stem cell transplant would be delayed while we use diuretics to restore their weight to the pre-mobilization level.

We have successfully reduced the transplant-related mortality rate to 1.1% [150] and continue to see good outcomes, including patients with cardiac amyloidosis before the onset of advanced congestive heart failure. Our hematologic and cardiac response rates are now approaching 66% and 41%, and hematologic response is directly connected to survival. We have reported our 10-year results and survival in patients who have received transplants and it is 43% [151]. At Boston University Medical Center, 10-year survival has been reported to be 53%, with mortality rates of 4–7%.

Currently, we are excluding patients from stem cell transplant who have an NT-proBNP level > 8500 or a systolic blood pressure < 90, suggesting that the extent of cardiac failure makes high-dose chemotherapy excessively toxic. Overall, we find 20–25% of all patients eligible for transplant. We are now seeing complete posttransplant responses of 39%. The most important predictor of survival is Mayo stage. The most important predictor of outcome, however, is maximal hematologic response. Some patients improve long before it would be considered possible for amyloid to be removed from the organ. This is particularly the case with cardiac amyloid and does suggest that immunoglobulin light chains have a direct toxicity to the heart, and this has been verified in isolated mouse muscle studies and in a *C. elegans* model of contractility [152]. Occasional second courses of

high-dose chemotherapy can be administered in those patients that had a durable first response. Induction chemotherapy plays an important role in the treatment of amyloidosis. In a prospective randomized study recently published, albeit small, the use of induction chemotherapy followed by transplant resulted in a better overall survival than transplant alone. Unfortunately, this trial was not stratified for the percentage of plasma cells in the bone marrow at the time of diagnosis and randomization. A recent review of the ABMTR registry identified 1536 patients with AL transplanted at 134 centers. The overall survival and early mortality were analyzed in three time cohorts: 1995–2000, 2001–2006, and 2007–2012. When available, both hematologic and renal response data was included. This trial showed that all-cause mortality at day-100 declined from 20% to 5%, with a 5-year overall survival improvement from 55% to 77% [145]. In the cohort study from 2007 to 2012, hematologic response to transplantation improved, and the renal response rate was 32%. This trial reported that centers that performed more than four AL transplants per year had superior outcomes, suggesting experience matters, and familiarity with myeloma transplant is not a surrogate for amyloidosis experience. In a multivariable analysis, cardiac AL was associated with the highest mortality and worst progression-free and overall survival. Higher doses of melphalan were associated with a reduced risk of relapse, although patients who received lower doses of melphalan are generally older and have greater degrees of cardiac and renal insufficiency. A creatinine of 2 mg was associated with a poorer overall survival [145].

In an observational trial of GCSF and plerixafor, the combination produced a higher total CD34 yield and a greater proportion of CD34 cells collected on the first day of apheresis. Four patients were mobilization failures with G but none with G and plerixafor, and patients who received plerixafor actually had a lower weight gain of 0.5 kg vs. 3.2 kg. The number of apheresis sessions, the number of hospitalization days, and cardiac arrhythmias were similar. This trial suggests that the up-front use of plerixafor results in a superior stem cell mobilization compared with G alone [153].

The British Amyloidosis Center reported on 90 patients who had undergone autologous stem cell transplantation over a 9-year period (38% as part of initial therapy). The responses, as measured by complete and very good partial response rates, were thought to be superior to those reported with low-dose chemotherapy. Renal responses were noted in 33% and liver responses in 7%; and out of 17 patients evaluable for a cardiac response, a cardiac response was seen in 6 (35%). The median progression-free survival was approximately 4 years, supporting the use of stem cell transplantation and its ability to produce deep and durable responses [154].

Oregon Health Sciences University reported on 31 patients receiving autologous stem cell transplant, including patients who were pretreated and those who had received prior induction chemotherapy; 13 patients proceeded directly to transplant after diagnosis, 12 received a bortezomib-containing regimen before transplant, and 6 had other variable induction therapies prior to transplant. The all-cause, day-100 mortality was 9.6%, and the hematologic and organ response rates were 77% and 58%, respectively. The median time to hematologic response after transplant was shorter in the group that received bortezomib induction, 3 vs. 14 months. The overall cardiac response rate was 60% (100% in those pretreated with bortezomib and 43% in those without induction treatment). The 3-year progression-free and overall survival was 66% and 73%, and they concluded that bortezomib-based induction was well tolerated both in patients with and without cardiac involvement and could be considered as part of the initial therapy of amyloidosis [155].

The European HOVON German Cooperative Group for the treatment of amyloidosis conducted a phase II trial evaluating three courses of vincristine, doxorubicin, and dexamethasone, followed by an autologous transplant; and 69 patients were transplanted between November 2000 and January 2006. After long-term follow-up of 115 months, the median survival of all patients was 96 months from registration and, for the transplanted patients, 120 months from the date of transplant. Therapy-related mortality was 12%. Four patients died during vincristine, doxorubicin, and dexamethasone, and two additional patients of the remaining 57 (or approximately 4%) died following high-dose melphalan. A two-step approach consisting of less toxic induction therapy followed by high-dose melphalan may result in extended overall survival [156].

The Mayo Clinic Group has now completed stem cell transplantations on a total of 663 patients. The profound effect of NT-proBNP on outcome is given in the figure where survival is split at the level of NT-proBNP of 1800 pg/mL (Fig. 36.9).

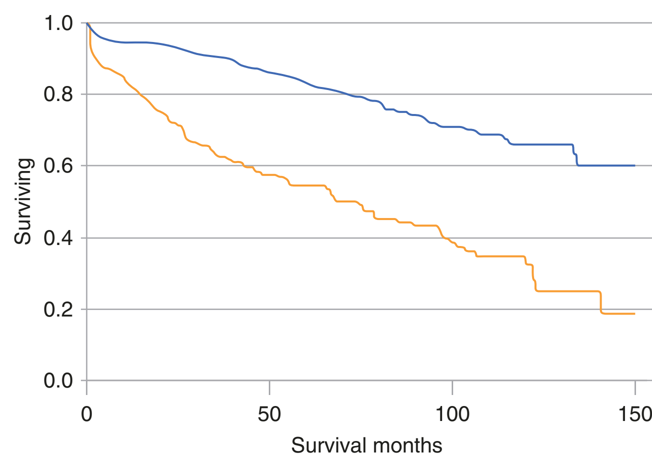

Fig. 36.9 Survival after stem cell transplantation stratified by NT-proBNP ≥ 1000 ng/mL. *Blue line*: NT-proBNP < 1000 ng/mL; *orange line*: NT-proBNP ≥ 1000 ng/mL

There is no reliable phase III data that demonstrates a survival advantage for stem cell transplantation. The only published phase III trial was plagued with poor patient selection and a very high therapy-related mortality [157]. Patients with AL, however, are highly selected for transplant. They are younger, have fewer organs involved, and will not have advanced heart failure. Therefore, concluding that stem cell transplantation is superior to conventional chemotherapy is not possible. The experience of most amyloid experts remains that if the patient is eligible for stem cell transplantation, they should be referred for therapy. Our current policy is to offer induction chemotherapy for those patients that have >10% plasma cells in the bone marrow. However, if the percentage of plasma cells is well below the threshold for multiple myeloma, then we are comfortable in going directly to stem cell transplantation and following the policy of Memorial Hospital in New York that uses bortezomib consolidation after transplant for those that have not, as yet, achieved a complete response [158].

Immunomodulatory Drugs

Thalidomide, lenalidomide, and, most recently, pomalidomide have been introduced for the treatment of immunoglobulin light chain amyloidosis, and all have shown significant activity. In the first thalidomide trial of amyloidosis, 16 patients had dose escalations to 300 mg/day. At this dose, treatment was poorly tolerated, and a high proportion had to abandon therapy before they had an opportunity to develop an organ response [159]. In a report of 12 patients with light chain amyloidosis treated with thalidomide, nine had severe drug-related toxicity and a median treatment exposure of only 72 days [160].

In Great Britain, thalidomide was combined with cyclophosphamide, thalidomide, and dexamethasone at only 100 mg/day, and 31% still had to discontinue therapy, although the hematologic response rate was 55%, and the treatment-related mortality was only 4%. Bradycardia can be seen in as many as 26% of patients. Therefore, care is required when this is administered to patients with cardiac amyloidosis [161]. A recent joint European study compared outcomes of bortezomib-based regimens with thalidomide-based regimens, and this retrospective multicenter review suggested that risk-adjusted bortezomib regimens were better than IMID-based regimens [162].

The second-generation immunomodulatory drug, lenalidomide, has a much better safety and toxicity profile in patients with amyloidosis [163]. It has been used with dexamethasone as well as with cyclophosphamide. The usual maximum-tolerated dose of lenalidomide is approximately 15 mg/day, but it can produce a response rate of 58%, 42 complete, with a 2-year overall survival of 81%. As with thalidomide, prophylaxis against venous thromboembolism is required. A combination of cyclophosphamide, lenalidomide, and dexamethasone is safe. No comparative trials exist to determine if it offers advantages to the doublet alone. Lenalidomide has been reported to increase the level of NT-proBNP and can actually aggravate heart failure; and the dose should not exceed 15 mg daily. The discontinuation rate of lenalidomide is high within the first three cycles. Skin rash, fatigue, and myelosuppression remain common. In a study of lenalidomide and dexamethasone in patients who failed melphalan and bortezomib, two (8%) died prior to first-response evaluation, 50% experienced grade 3 or greater toxicity, and the hematologic response rate overall was 41%. The median overall survival was 14 months. When cyclophosphamide was added in doses of 100 mg/day, the hematologic response rate was 55%. Lenalidomide was associated with fatigue and fluid retention. When amyloidosis, refractory to both melphalan and bortezomib, was treated with lenalidomide and dexamethasone, 24 patients were enrolled; 19 were refractory to thalidomide, two died before response evaluation, and the adverse event rate was 50%. Survival was shorter in patients with an elevated troponin and in those patients diagnosed <18 months before treatment initiation. Hematologic response rate was 41%, with a median overall survival of 14 months [164].

Melphalan and dexamethasone have been combined with lenalidomide; and in a phase I dose escalation, an MTD of 15 mg was achieved. Melphalan dose for amyloid patients was scaled back due to their frailty at 0.17 mg/kg/day for 4 days. In this trial, dexamethasone was given in a little-used schedule of 40 mg on days 1–4 every 28 days. All patients received low-molecular-weight heparin for DVT prophylaxis; 26 patients were evaluable, and 6 deaths were seen. The complete response rate was 42%, PR rate was 9 of 26, and an overall response rate was 58%, with 50% organ responses, a 54% event-free survival of 2 years, and an overall survival of 81% at 2 years [165].

When melphalan, dexamethasone, and lenalidomide was used in advanced cardiac amyloidosis, 25 patients were enrolled at a dose of 0.18 mg/kg/day for 4 days and dexamethasone 40 mg once weekly with lenalidomide 10 mg/day. Early cardiac deaths were seen in 42% of patients, arrhythmias in 33%, and the hematologic response rate was 58%. Overall survival at 1 year was 58%, but cardiac response rate was only 9%; and the median overall survival for patients with heart failure was 1.75 months [166].

Lenalidomide, dexamethasone, and cyclophosphamide were given to patients for 12 cycles, two-thirds of whom had no prior therapy. The MTD for lenalidomide was 15 mg, and

the MTD for cyclophosphamide was 100 mg/day. The PR rate was 55%, the CR was 8%, and four of five prior bortezomib patients responded. In 6-month survivors, the organ response rate was 40%, and the 2-year overall survival rate was 41%. A trial in newly diagnosed amyloidosis with cyclophosphamide, lenalidomide, and dexamethasone ended up enrolling a total of 41 patients. The ≥VGPR rate was 43%. Organ responses were seen in 23 heart, 23 kidney, and 4 liver patients. The overall and progression-free survival in those patients who achieved a PR or better was highly significant ($p < 0.001$).

Bortezomib-Based Chemotherapy for AL Amyloidosis

Eighteen patients, including seven who had relapsed or progressed, were treated with bortezomib and dexamethasone; 94% had a hematologic response, 44% complete, five patients (28%) had a response of at least one affected organ, first establishing activity of bortezomib in amyloid [167]. Our report on bortezomib in 20 patients with active clonal disease despite prior therapy, including thalidomide, was reported. Three (15%) achieved completed hematologic response, and a further 13 (65%) achieved a partial response, but 75% experienced toxicity, requiring discontinuation in 40% [168]. The first published study of weekly and twice-weekly bortezomib that was prospective reported hematologic responses in 50% of 30 evaluable patients, including 20% complete responses with a median time to response of 1.2 months with no difference between once-week and twice-weekly bortezomib [169]. A multicenter survey on the use of bortezomib across Europe reported on 94 patients from three centers. Previously untreated patients had a 47% complete response rate, and a 1-year survival was 76% with the NT-proBNP independently associated with survival [170]. In a series of 26 patients treated with bortezomib and dexamethasone, 69% received it as first-line therapy. The overall response rate was reported at 54%, with 31% hematologic complete responses. Median progression-free and overall survival was 5 and 18 months, respectively, but not reached in complete-response patients [171]. Bortezomib has also been used following autologous stem cell transplant as back-end consolidation for patients who do not achieve a complete response and was demonstrated to eradicate clonal disease at 12 months post stem cell transplantation. Consolidation with bortezomib achieves high stringent complete response rates [158]. An update of the original phase I-II trial conducted in Canada showed 1-year hematologic progression-free rates of 72.2 and 74.6 in patients receiving weekly and twice-weekly bortezomib. Organ responses included 29% renal and 13% cardiac [172].

The combination of cyclophosphamide, bortezomib, and dexamethasone was administered to 17 patients; a response was seen in 16 (94%), 71% achieving a complete hematologic response, and 24% a partial response. Three patients originally not eligible for stem cell transplantation became eligible, and this has become the standard of care at Mayo Clinic [173]. Bortezomib has also been combined with cyclophosphamide and dexamethasone, and the patients who received this at initial therapy had complete response rates of 65% with a 2-year progression-free survival of 66.5% and an overall survival at 2 years of 97.7% [174]. A confirmatory trial of cyclophosphamide, bortezomib, and dexamethasone as first-line treatment reported an overall response rate of 93% with 59% achieving a very good partial or complete response with no grade III-IV peripheral neuropathy [175]. Induction therapy with bortezomib and dexamethasone followed by autologous stem cell transplantation resulted in better outcomes than patients who went directly to stem cell transplantation without bortezomib therapy, achieving statistically significant improvement in overall survival at 24 months (95% vs. 69.4%) [176].

Bortezomib, cyclophosphamide, and dexamethasone have also been shown to be effective in high-risk cardiac amyloidosis (Mayo Clinic stage III). The overall response rate in this cohort was 68%. The estimated 1-year survival was 57%, 40% dying on therapy. Although unable to save the poorest-risk patients, bortezomib, cyclophosphamide, and dexamethasone achieved a high number of hematologic and cardiac responses [177]. Bortezomib has been combined with melphalan and prednisolone in the treatment of newly diagnosed AL amyloidosis. Among 19 patients enrolled in the trial, 16 (84%) had a hematologic response, including 37% complete responses with cardiac and renal responses in 44% and 33%, respectively [178]. In a matched-case control study of melphalan and dexamethasone with or without bortezomib, a higher rate of complete responses was observed with bortezomib (42% vs. 19%), but this did not result in a survival improvement for the overall population [140]. Induction bortezomib followed by high-dose melphalan and autologous stem cell transplantation was reported in 12 patients. The day-100 treatment-related mortality was 9.6%. Hematologic and organ response rates in the entire cohort were 77% and 58%, respectively. Overall cardiac response rate was 60%. The 3-year progression-free and overall survival rates were 66% and 73% [155]. A matched comparison of cyclophosphamide, bortezomib, dexamethasone with cyclophosphamide, thalidomide, and dexamethasone reported that the bortezomib-containing combination improved depth of response and resulted in a superior progression-free survival, supporting its use as opposed to a thalidomide-based therapy [161]. Translocation 11;14 is

associated with a poorer outcome in patients treated with bortezomib-based regimens. The reason for this negative impact of this translocation remains uncertain [15]. Bortezomib-based induction for transplant and eligible amyloidosis has resulted in a significant improvement to allow patients to undergo transplant; and in one trial, 8 of 24 initially transplant-ineligible patients became eligible, 7 of whom achieved sustained hematologic response 33 months posttransplant [179]. An update of long-term outcomes after bortezomib therapy of patients reported a median survival of 47 months in patients treated with bortezomib. Bortezomib was associated with improved 1-year survival compared to lenalidomide therapy (81% vs. 56%) [162]. A European collaborative report of cyclophosphamide, bortezomib, and dexamethasone reported 230 patients treated with front-line cyclophosphamide, bortezomib, and dexamethasone. Overall hematologic response rate was 60%, cardiac responses in 17%, and renal responses in 25% [180]. Induction therapy with bortezomib followed by bortezomib and melphalan conditioning for autologous stem cell transplantation was applied to 27 patients, and 100% hematologic responses were seen, 37 very good partial responses; and with a median follow-up of 36 months, median overall and progression-free survival had not been reached [181].

Seventy-three consecutive unselected patients were treated with first-line bortezomib-based induction. Hematologic responses were seen in 77%, including 33% very good partial responses. First-line bortezomib resulted in favorable response and survival [182]. In conclusion, it appears that bortezomib is the single most active agent available for the treatment of amyloidosis. A current randomized phase III trial looking at the oral proteasome inhibitor, ixazomib, is underway.

Antibodies

Anti-plasma-cell-directed chemotherapy does nothing to address established amyloid deposits. Three monoclonal antibodies are currently being tested for efficacy in the treatment of amyloidosis. The 11-1F antibody is currently undergoing clinical testing for efficacy. A second antibody against serum amyloid P component has been shown to improve the amount of amyloid in the liver and spleen and has resulted in improved ultrasound characteristics of the liver. Finally, the Neotope antibody has been given to 27 patients who no longer required anti-plasma-cell chemotherapy; and of 14 cardiac evaluable patients, 57% met the criteria for cardiac response; and of 15 renal evaluable patients, 60% met the criteria for renal response. This antibody, NEOD001, is now undergoing a global phase III trial [183].

Conclusion

Light chain amyloidosis needs to be considered in the differential diagnosis of patients with nondiabetic nephrotic range proteinuria, unexplained fatigue or heart failure with preserved ejection fraction, a progressive peripheral neuropathy associated with a monoclonal protein, or atypical multiple myeloma. Screening involves serum and urine immunofixation and an immunoglobulin free light chain assay. Most patients can have the diagnosis established via biopsy of the bone marrow and subcutaneous fat. Visceral organ biopsy is rarely needed. The prognosis is determined by the DFLC, the levels of cardiac biomarkers, troponin and BNP. Therapy includes high-dose chemotherapy; and for patients ineligible for high-dose chemotherapy, bortezomib appears to be the single most active agent (Fig. 36.10). Combinations of bortezomib and the inclusion of monoclonal antibodies are the subject of further studies.

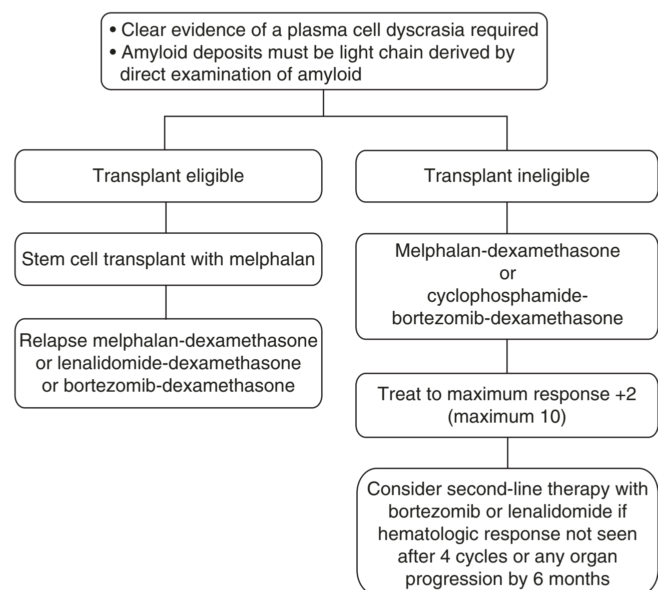

Fig. 36.10 Algorithm for amyloidosis therapy

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Part IV

Lymphoma

Check for updates

Historical Landmarks in an Understanding of the Lymphomas

37

Marshall A. Lichtman

Ancient Oncology

Cancer, generically, has been an affliction of both simple and complex species, presumably since their evolution, and studied most exhaustively, although not exclusively, in mammals [1]. The presence of cancer in ancient times has been suggested by holes in bones found in ossuaries or in the remains of mummies, which in some cases have been thought to be lesions indicative of myeloma. Metastatic cancer was considered if the lesions had osteoblastic features and myeloma was thought probable if osteolysis alone was present [2, 3]. Differences in reproductive patterns, life expectancy, diet, tobacco and alcohol use, and exercise should lead to differences in cancer prevalence today, compared to the frequency in ancient societies. One study has concluded that, corrected for sex and age distributions of ancient populations, Upper Egyptian societies, circa 3200 to 500 B.C.E., Southern German populations, circa 1400 to 1800 C.E., and British populations, circa 1901 to 1905 C.E., may have had similar frequencies of cancer as in modern times [2]. The increase in the incidence of cancer in modern times was ascribed largely to longer life spans. Paleo-pathology, or, more specifically, paleo-oncology, is fraught with the challenge of artifacts, some of which occur postmortem and do not reflect antemortem disease; thus, the conclusion that lesions found in mummies in societies that preserved corpses (e.g., Egyptian, Incan, Papua New Guinean) represent the residue of cancers has been challenged [2–4]. Soft tissue cancers, such as lymphoma, would be very hard to discern after thousands of years of internment, but cancers have been described in Egyptian and Peruvian mummies. Our current understanding of the development of somatic mutations from errors in cellular DNA replication or repair, neoplastic clone formation, clonal selection, and clonal evolution to a progressive neoplasm underlies the knowledge that cancer is an invariable coincidence of the evolution of multicellular organisms from time immemorial [1, 5, 6].

Breast cancer is the best documented soft tissue cancer that afflicted ancient societies, in part because of its location. Cancer of the breast and its treatment by cauterization were described in the Edwin Smith (1822–1906) papyrus written circa 1600 B.C.E., the only surviving copy of part of an ancient Egyptian medical textbook and one of the world's earliest surviving examples of medical literature, held by the New York Academy of Medicine. It indicates a rather rational approach to problems for that era. The Kahun gynecological papyri, considered the oldest of the medical papyri, dated to circa 1800 B.C.E., deals exclusively with diseases of women, fertility, contraception, and pregnancy. Other disease descriptions are found in the Georg Moritz Ebers (1837–1898) papyrus of Thebes, dated to circa 1550 B.C.E. held in the library of the University of Leipzig. Similar medical descriptions have been found in recovered ancient Persian and Hindu literature. Ancient cultural views thought of cancer as the result of evil spirits, astrological events (e.g., the disharmonious movements of celestial bodies), violation of religious rules, and, generally, the anger of the gods. Thus, incantations and mystical approaches to disease are described, especially in the Ebers papyrus. However, arsenical ointments and other escharotics were used by the ancients for cancer therapy. Although there is no specific information available relevant to lymphoma, given our current understanding of de novo cancer development, as an inevitable

Note: Many of the English, Scottish, and Irish physicians or scientists discussed in this chapter received a knighthood and the appellation "Sir" could be used. Since they virtually all did their seminal work before they had been knighted, I have not identified them as "Sir" in the chapter.

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consequence of errors in DNA replication and repair, malignancies of lymphocytes surely occurred in antiquity. Their frequency may have been much lower, since lymphoma incidence increases with age and lifespan was shorter. Also, the incidence of lymphoma increased significantly in the twentieth century. It has been postulated that modern methods of hygiene and antibiosis prevent postnatal modulation of the immune system, an evolutionary requirement to ensure normal function. The absence of modulation, induced by untreated, more frequent bacterial infections in childhood, may be a determinant of the increased frequency of lymphoid malignancies in First World countries in modern times.

It was over 1000 years after the Ebers papyrus was written that Hippocrates (460 to 370 B.C.E.) used the Greek term "carcinos" to describe tumors and "carcinoma" to describe ulcerating tumors. The root "carcino" is derived from the Greek for crab. The reason for this derivation is unknown, but it has been speculated that Hippocrates may have drawn an analogy between the limbs of a crab and the invasion by radial extensions of a cancer into tissues. Hippocrates also described cautery to remove skin cancers. Aulus Cornelius Celsus (circa 28 B.C.E. to 50 C.E.), a Roman, translated the Greek word "carcinos" into the Latin term "cancer." Celsus was a Roman encyclopedist and medical historian. His encyclopedia, *De artibus*, contained five books on agriculture, and other books on government, history, law, medicine, military techniques, philosophy, rhetoric, and science. Only *De medicina octo libri (The Eight Books of Medicine)*, the most comprehensive medical history and detailed description of medical and surgical procedures ever produced by a Roman writer, has survived. Celsus, for example, wrote about the appropriate surgical approach to mastectomy for breast cancer, a technique that spared the pectoralis muscles. Aelius (a.k.a. Claudius) Galenus (Galen) (131–203 C.E.) was born in the Greek city of Pergamum in Asia minor (current day Turkey). He introduced the term "onkos" from the Greek meaning mass or tumor, to refer to a malignant growth, and he espoused the concept of accumulation of black bile in tissues as the immediate cause of cancer. The latter concept was spawned by the Aristotelian division of the physical world into four elements: earth, air, fire, and liquid (water). Galen conducted anatomical studies on monkeys, did nerve ligation studies, had an interest in arteries and veins, and used the pulse rate as an indicator of disease. Some consider him the father of experimental medicine. Unfortunately, Galen had his imagination but few scientific tools to apply to his interests. He believed that food decomposed in the bowel was transported to the liver by the sucking ability of the vessels, and in the liver was converted to blood, which was transported to the heart by hepatic veins and from there only to the lungs for their sustenance. These concepts lead Galen to conclude that blood was the direct derivation of food and quantitatively related to the amount of food ingested. Galen theorized that four fluids, blood, phlegm (mucous), yellow bile, and black bile governed bodily processes and the balance or imbalance among them caused disease. He thought that black bile was formed in the spleen as a result of undigested food reaching it through a canal from the stomach. These concepts dominated medical thinking for the next 1200 years.

This inability to apply logic, anatomical dissection, and experimentation to medicine, in part, was the result of the prohibition by early cultures and, later, the Church to dissecting the human body or to study or use surgical procedures, leaving monks to translate and propagate the misconceptions of Galen and the application of alchemy, astrology, and magic to the causation of human disease. Even the opening of the universities in Paris and other principal European cities at the end of the first millennium C.E. did not significantly advance learning about human physiology or medicine because the Church at that time dictated the books that could be used in those institutions.

Theophrastus Phillippus Aureolus Bombastus von Hohenheim (a.k.a. Paracelsus) (1493–1541) was one of the first to challenge the Galenic theory of disease. Paracelsus was used as his pseudonym and meant "equal to or greater than Celsus." Paracelsus was a Swiss physician and chemist who derided Galen's views on disease causation and suggested sulfur or arsenic accumulated in the blood, especially in miners, masons, metal workers, and others exposed to potential toxins, which led to cancer. Ambroise Paré (1510–1590), the renowned French surgeon and expert on battlefield wounds, argued that an inappropriate diet resulting in feculent material accumulating in the blood was the immediate cause of cancer. Although both were considerably off the mark, one can see a shift from unhappy gods to more relevant precedents, such as exposure to environmental toxins or inadvisable ingestants. Paracelsus, in part because of his argumentative, adversarial, and zealous approach to controversy, incited the Galenists who beat him to death.

The development of the printing press and the environment for observation and discovery defining the Renaissance made possible the propagation of the remarkable anatomical studies of Andreas Vesalius (1514–1564) in his seven-volume masterpiece, *De Humani Corporis Fabrica Libri Septem* (*On the fabric of the human body in seven books*). The influence of René Descartes (1596–1650) who, in the eternal dispute between faith and reason, favored questioning everything until one reached the strength of fact, established the foundation of the scientific method, and the distrust of pontification. Descartes' publication *Discours De La Methode Pour bien conduire sa raison, & chercher la verité dans les sciences…* (*Discourse on the Method of Rightly Conducting the Reason and Seeking Truth in the Sciences…*) was, perhaps, the most influential publication of the seventeenth century, providing a framework for scientific reasoning.

Isaac Newton (1643–1727), the English mathematician, astronomer, and physicist, published *Philosophiæ Naturalis Principia Mathematica* (*Mathematical Principles of Natural Philosophy*), often referred to as *Principia*, in 1687. It was the basis for the field of mechanics, describing universal gravitation and the laws of motion and providing the scientific perspective of the physical universe for the next 300 years. Newton showed that the motions of objects on Earth and of celestial bodies are governed by the same set of natural laws by demonstrating the consistency between Johannes Kepler's (1571–1630) laws of planetary motion and Newton's theory of gravitation, thus confirming, indisputably, the concept of heliocentrism. Buoyed by these great achievements in the physical sciences, the studies of Malpighi, Morgagni, Harvey, and other great experimentalists of the day, in fields related to biology and medicine, encouraged scientists to challenge earlier misguided ideas about cancer and its causes and to pursue new avenues of inquiry using dissection, microscopy, clinical description, classification, and other techniques to which could be applied the developing ideas about the scientific method of inquiry (covered later in this chapter).

The Introduction of Microscopy and of Examination of Tissues

The invention of the microscope was essential for the furtherance of knowledge about the cellular pathology of cancer and was among the most significant inventions leading to our advanced understanding of the lymphomas. At the end of the sixteenth century, a Dutch spectacle maker, Sacharias (Zacharias) Jansen (Janssen) (c.1580–c.1638), discovered that nearby objects appeared greatly enlarged when examined through lenses in a tube. His simple and crude device, using an eyepiece lens and an objective lens, was the forerunner of the modern compound microscope. In 1609, Galileo Galilei (1564–1642), the Italian physicist, mathematician, astronomer, and philosopher, considered the father of modern science, learned of Jansen's experiments and, so stimulated, developed the principle of lenses and made an improved compound microscope that included a focusing device. Galileo's contemporary Giovanni Faber (or Johann Faber) (1574–1629) was a German botanist who moved to Rome in 1598. He was curator of the Vatican botanical garden, a member and the secretary of the Accademia dei Lincei ("Academy of the Lynx-Eyed"), anglicized as the Lincean Academy, in Rome. Faber coined the term "microscope" from the Greek words "micron" meaning "small," and "skopein" meaning "to look at." The Academy, founded in 1603, was one of the first academies of science to exist in Europe and was named after the lynx, an animal whose sharp vision symbolizes the observational skill required by scientists.

The Dutchman Antonj van Leeuwenhoek (1632–1723) was an apprentice in a linen-draper shop in Delft where he was being instructed in the use of magnifying glasses to count the threads in cloth. Later, he married and opened his own draper shop. His early experience resulted in his attempts to develop new methods for grinding and polishing lenses of a curvature that were capable of magnifications of nearly 300-fold. Using these monocular magnifying devices of his construction, he described the appearance of bacteria, the first description of spermatazoa, and other microscopic findings, and published his observations in several hundred letters to the Royal Society of England and the French Academy. Leeuwenhoek, the first to describe single cell organisms (i.e., bacteria), which he called "animalcules," was initially disbelieved, as there was no concept of living organisms of these dimensions. The Royal Society sent a group of men to Delft to verify these findings. When verified, the observations required the Society (and the world) to revisit its theory of living organisms. Robert Hooke (1635–1703), an Englishman, confirmed many of Leeuwenhoek's observations, lauded his pioneering work, and improved the design of the light microscope. Hooke wrote *Micrographia*, the first book published on the topic of microscopy in which he described his microscopical observations. He was the first to use the word "cell" to identify microscopic structures when studying the structure of cork.

By the mid-nineteenth century, European countries began to manufacture fine optical equipment including microscopes. Advances were made in the nineteenth century that removed disturbing artifacts (e.g., bright halos), which interfered with the visualization of objects. The development of the achromatic lens by the specific fusion of a convex and concave lens eliminated chromatic and spherical distortions. Other improvements were accomplished by determining preferred combinations of lenses and the distance between lenses that resulted in an optimal image. These advances were paralleled by improved methods of preserving, hardening, cutting, and staining tissue for examination. Among the finest microscopes of the day were those built by the American Charles A. Spencer (1813–1881) living in relative isolation in a small village in central New York State; they provided magnification of nearly 1000-fold and were deemed the equal of or better than the finest English or Continental models. Spencer, self-taught, was one of the finest opticians of his day. In Europe, Carl Zeiss (1816–1888) opened an optics workshop in Jena, Germany, in 1846. By 1861, Zeiss was considered to be among the best lens and scientific instrument makers in Europe. The lenses he was manufacturing in addition to advancing microscopy also propelled photography.

Even with these superb light microscopes, structures less than half the wavelength of light, 0.275 mm, were not resolvable. It required about 100 years to advance microscopy to

see smaller objects and structures. In the early 1930s a team in Germany had been able to bypass light and use a different form of illumination, an electron beam accelerated in a vacuum. In 1931, working closely with Max Knoll (1897–1969), Ernst Ruska (1906–1988) built the first electron lens, an electromagnet that could focus a beam of electrons as if it were a light source. His prototype of an electron microscope could magnify only 17-fold, but he had accomplished proof-of-principle. Within 2 years, Ruska's electron microscope, which he termed a "transmission microscope," was much improved. An image was generated by passing electrons through a thin specimen and then deflecting them to a photographic film emulsion or projecting them onto a fluorescent screen, generating an image at high magnification. This device was capable of magnifying specimens up to approximately 10,000-fold. Ruska temporarily joined the Siemens Company in Germany[1] as an electrical engineer in 1937 and the company released its first commercial electron microscope in 1939, based on Ruska's design. When Ruska received the Nobel Prize in Physics in 1986 for his work, the Nobel Committee described the electron microscope as one of the most important innovations of the twentieth century. By that time further improvements of the device had made it capable of magnifying an object up to one million times its size, making it possible to view viruses and molecules. The electron microscope made possible the discovery of the Epstein–Barr virus (see later discussion in this chapter) among innumerable other findings important to the advanced understanding of cancer in general and lymphoma in particular.

Early Views of the Lymphatic System and Cancer

The lymph nodes had been described by the last third of the seventeenth century by the pioneering physician, embryologist, and histologist Marcello Malpighi (1628–1694). Malpighi was an extraordinary observer and the earliest biological microscopist. Although the compound microscope was available since the late sixteenth century, Malpighi was the first to use it with such significant results.

His discoveries in human and animal biology and botany were such that he was made a member of the Royal Society of England, the first Italian to be so honored. Many of his findings were published as letters to the Royal Society. Although William Harvey (1578–1657) predicted the existence of capillaries when he announced his discovery of the pulmonary and systemic circulatory systems in 1616, fully described in his monumental treatise, *Exercitatio Anatomica de Motu Cordis et Sanguinis in Animalibus* (An Anatomical Exercise on the Motion of the Heart and Blood in Living Beings) published in 1628, Malpighi observed blood coursing through them, having the advantage of the application of microscopy to his work. In an undated letter from Malpighi to Prince Marcantonio Borghese (circa 1687), he gave his detailed description of the conglobate glands, which included the lymph nodes, into which lymphatic vessels flowed and exited. He refuted the idea that they were fibrous swellings, as had been suggested by others. He also described the white pulp of the spleen, the splenic lymph nodules, which were later named Malpighian corpuscles in recognition of his landmark studies of splenic structure [8]. He did not appreciate that they were lymphatic in nature, but identified them as separate, glandular structures. Malpighi also recognized diseases that led to gross enlargement of lymph nodes and spleen and he may have been the first to describe cases of lymphoma. Malpighi and Morgagni described the histopathology of cases that were felt by David Craigie (see later discussion in this chapter) to have been the earliest descriptions of Hodgkin's disease.

Giovanni Battista Morgagni (1682–1771) was an Italian anatomist, and the earliest of the anatomical pathologists. He had studied with Antonio Maria Valsalva (1666–1723), who had been a student of Malpighi. He became the professor of anatomy at Padua. In 1761, when he was 80 he published his great work, *De Sedibus, et causis morborum per anatomem indagatis libri quinque* (The seats and causes of disease, investigated by anatomy, in five books), which made pathological anatomy a science and enhanced the precision of medical practice. His treatise was based on the study of hundreds of case histories and autopsies performed at the University of Padua. His proposal that disease was caused by a malfunction in bodily organs provided a rational substitution for the ancient concept of ill humors, black bile, and other irrelevancies as a cause of disease, a revolution in the understanding of illness. His association of exaggerated fat accumulation at autopsy with several diseases established today as related to obesity is an example of his perceptive analyses. During the succeeding 10 years, his treatise was reprinted several times in Latin (its original language) and translated into French, English, and German. Although Andreas Vesalius, Gabriele Falloppio (1523–1562), Geronimo Fabrizio (a.k.a. Hieronymus Fabricius) (c1533–1619), and others in prior generations had described normal

[1]At this period, the Siemens company (and other German industries) had abetted the rise of the National Socialist Workers Party (commonly called the Nazi party) and supported the policies of Adolph Hitler (1889–1945). A few years later, Siemens built electronics factories in the close vicinity of concentration camps and used slave labor in its plants. The company was complicit in the worst aspects of the Third Reich's inhumane policies and its execution of the Holocaust. Siemens ran factories at Ravensbrück (a camp limited to women prisoners at which brutality and vile and grotesque medical experiments were the norm) and in the Auschwitz subcamp of Bobrek among others [7].

human anatomy carefully, indeed brilliantly, and, thereby, to debunk such concepts as Galen's canal connecting the stomach to the spleen, Morgagni was the first to make pathological anatomy a central approach to medicine.

The milky fluid of lacteals was described in the mid-seventeenth century. Jean Pecquet (1622–1674), a French physician, described the cisterna chyli, the thoracic duct, and observed that the thoracic duct entered into the large veins of the upper thorax in the dog. In Denmark, Thomas Bartholin (1616–1680) confirmed these findings in humans and observed that they formed a separate vascular system and named them "lymphatics" and their content "lymph" (from the Latin, *lympha*—meaning "clear spring water"). It had been speculated that they arose from arteries, but Bartholin provided evidence that there were three systems of vessels: arteries, veins, and lymphatics.

For nearly 175 years during the sixteenth and seventeenth centuries, some observers thought that lymph nodes were the coiled mass of lymphatic vessels. The afferent lymphatics were thought to enter the gland over its convex surface and become intertwined to make up the bulk of the gland and then exit as efferent lymphatics. William Hewson (1739–1774), the son of a country surgeon, was trained in medicine as an apprentice to a surgeon in Newcastle-on-Tyne [9]. After studies in Edinburgh and Paris, in 1759, he went to William Hunter's (1718–1783) preeminent school of anatomy in London; he also studied at St. Thomas's and Guy's hospitals. During 1768–1769, Hewson read three papers to the Royal Society on his exploration of the lymphatic system in the lower vertebrates (birds, amphibia, and fish), and made a more complete demonstration of the lymphatic system than his predecessors. He was elected a fellow of the Royal Society in 1770. He provided the first account of coagulation of the blood and showed that it was the result of changes in plasma rather than cellular constituents. The compound microscopes at that time produced distortions and the procedures for preparing tissue for examination were inadequate. Hewson relied on a single lens and devised a satisfactory means of mounting "wet" specimens. In 1771, he published "An Experimental Inquiry into the Properties of the Blood, and an Appendix Relating to the Discovery of the Lymphatic System" in the *Philosophical Transactions of the Royal Society*, London. He gave the first good description of the various types of granular and agranular leukocytes. He was the first to observe the lymphocytes in the thymus and spleen (recognizing for the first time that the splenic corpuscles of Malpighi were lymphatic in nature) and concluded that their production was the intrinsic function of the thymus, lymph nodes, and spleen. The lymphocyte was so named because it was the principal cell found in the thin fluid, the lymph. He wrote, "By the lymphatic system and its appendages we mean the lymphatic vessels, the lymphatic glands, the thymus and the spleen." He recognized that the thymus provided lymphocytes "in the fetus and the early part of life." He reported his observations on the red corpuscles in 1773, showing that they were "flat as a guinea," not spherical, as was believed because of prior observers mistaking the center of the disk for a nucleus, probably an artifact of the primitive lens systems in use. He died from the effects of an infected dissection wound in 1774.

During the sixteenth through eighteenth centuries, a series of hypotheses about the nature of cancer developed, in part, related to the lymph. Cancers were thought to be contagious, thought to arise from coagulated lymph, thought to result from lack of movement of tissues (atony), and thought to result from the abnormal fermentation of blood and lymph. Despite these primitive and fallacious concepts, there was a steady progression among leading physicians of the day toward a cellular theory of cancer and the concept that cancers developed, locally, in a tissue related to the particular type of cancer formed. For example, in 1775, Percivall Pott (1714–1788) reported an association between exposure to soot in chimney sweeps and the incidence of scrotal cancer (later determined to be squamous cell carcinoma). He was the first person to demonstrate that a malignancy could be caused by an environmental carcinogen. Pott also showed that the cancer could be cured by prompt excision. Pott was one of the most respected surgeons of his day and published many other groundbreaking papers including his piece on fractures and dislocations. In London, in 1777, Bernard Peyrilhe (1735–1804) published the first treatise on cancer in Latin (*Dissertatio academica de cancro*), which was translated into English (*Dissertation on Cancer*). The publication highlighted the increasing exploration of and attempt to better understand this disorder. Peyrilhe was convinced that a microbe caused cancer and that a tumor's growth simulated the growth of a hen's egg into a chicken by incubation, requiring heat, humidity, perhaps obstructed blood vessels, and other factors.

The progress in this age was particularly notable in the life and works of Johannes Peter Müller (1801–1858). Müller became professor of anatomy and physiology and director of the Museum of Comparative Anatomy at the University of Berlin. He built a school that became famous and his students, who included Hermann Ludwig Ferdinand von Helmholtz (1821–1894), Theodor Schwann (1810–1882), Friedrich Gustav Jakob Henle (1809–1885), and others; it dominated German medical science in the second half of the nineteenth century. His *Handbuch der Physiologie des Menschen* published in parts between 1833 and 1840 was translated into English (*Handbook of Human Physiology*) and published in London in 1842. It was the leading textbook on the subject of human physiology and was revised and re-published several times. It was based on new concepts derived from his study of human and animal anatomy, chemistry, and other physical sciences, applying them to physiological

problems. He made extensive use of microscopy of pathological tissue and, in 1838, published a volume on the pathology of tumors. His work greatly influenced the young Rudolph Virchow. Müller's student Schwann introduced the "cell theory," declaring that human and animal tissues are the aggregate of cells, the building blocks of tissues.

In 1858, Rudolf Virchow (1821–1902), a German physician, anthropologist, and biologist, considered the father of human pathological study, published *Die Cellularpathologie in ihrer Begründung auf physiologische und pathologische Gewebenlehre* (*Cellular Pathology as Based upon Physiological and Pathological Histology*), a compendium of Virchow's 20 lectures delivered at the newly opened Pathological Institute of the University of Berlin [10]. Lecture VIII was entitled "Blood and Lymph." The lectures were dutifully recorded by a stenographer and Virchow used these verbatim transcripts to reproduce the lectures in his treatise on cellular pathology. Virchow extended concepts of disease from organs and tissues to cells. He opined, "All cells come from other cells… Where a cell originates it must have been preceded by another cell, just as animals are produced only by other animals and plants by other plants." He, also, proposed that disease is the result of disordered cellular function, a revolutionary concept.

Most of the major organs forming the lymphatic system (e.g., thymus, spleen, tonsils, lymph nodes) had been identified and unified as the site of production of the "colorless corpuscles" by Hewson and others well before Virchow's entry into medicine (discussed earlier in this chapter). The essential structures of lymph nodes and lymphatics were known and the structures of cortical lymph node follicles were interpreted as being similar to those of Peyerian patches, lymphatic nodules in the intestines, and the Malpighian corpuscles, lymphatic nodules in the spleen. Johann Conrad Peyer (1653–1712) was a Swiss physician and anatomist who had studied medicine in Paris and Montpellier, ultimately receiving his medical degree from the University of Basel [11]. He had observed the intestinal lymph follicles in 1673 and, in 1677, he published his findings under the title, *Exercitatio anatomico-medica de glandulis intestinorum earumque usu et affectionibus* (an anatomical-medical study on the intestinal glands, their functioning and diseases), which described the aggregated nodules found in the lining of the terminal small intestines, later to be named Peyer's patches. It, also, was later that they were determined to be part of the lymphatic system. Peyer who was an expert on the gastrointestinal tract thought they were digestive glands that emptied into the intestines through a duct (à la the pancreatic duct). Despite the fact that these intestinal lymphatic aggregates were first commented on by Marco Aurelio Severino (1580–1656), professor of anatomy and surgery in Naples, they were named for Peyer.

By the turn of the twentieth century, the essential description of the histological features of lymph nodes was incorporated into text books. For example, *The Principles of Pathological Histology* by Harvey Russell Gaylord (1872–1924), a professor of surgical pathology at the University of Buffalo, and Karl Albert Ludwig Aschoff (1846–1942), professor at the Pathological Institute at the University of Göttingen at that time, was published in 1901. Gaylord became the second director of what is today the Roswell Park Cancer Institute. In 1897, Dr. Roswell Park (1852–1914) and Mr. Edward H. Butler (1850–1914), publisher of the Buffalo Evening News, asked the New York State Legislature to introduce a bill that would provide a $7500 grant to establish a cancer research laboratory at the University of Buffalo School of Medicine. That bill was passed in 1898, and the New York State Pathological Laboratory of the University of Buffalo, the first facility in the world dedicated specifically to cancer research, was founded. The facility, which gave birth to what later would be known as Roswell Park Cancer (né Memorial) Institute, in honor of Buffalo's most prominent surgeon, has been cited as the earliest example of direct government support of cancer research. Gaylord and Aschoff's description of the histology of lymph nodes could just as well be in a histology text today [12]. However, there was no mention in the section of diseases of lymph nodes of lymphoma, Hodgkin's disease, or pseudoleukemia, the latter term used to encompass apparent malignant lymphoid diseases in which the white cell count was not prominently elevated. During the nineteenth and at the turn of the twentieth century, pseudoleukemia was one synonym for lymphoma and Hodgkin's disease, especially in Germany and some other nearby European countries (pseudoleukämie). The section of their text describing diseases of the spleen also had no discussion of lymphoma, lymphosarcoma, or Hodgkin's disease, indicating the slow and cumbersome means of spreading medical knowledge at that time. The changes of splenic histology with "leukemia" were described, but not in terms of specific leukemic types.

The Acceleration of Invention and Scientific Progress in the Eighteenth and Nineteenth Centuries

Advances in science and medicine, as today, are often the result of advances in industry, technology, the response to epidemics, and the demands of armies for better battlefield care of the wounded. The United States had achieved nationhood and was expanding westward fulfilling the principle of "Manifest Destiny." Its commercial and scientific achievements were to be unrivaled as a result of the rise of what would be the most innovative, industrious, and scientifically

oriented country in history, in large measure as a result of its openness to immigration of those constrained by autocratic governments, religious persecution, and nonprogressive societies and by its vigorous adherence to the separation of church and state. In France, the Monarchy had been replaced by a Republic of sorts. Antoine-Laurent de Lavoisier (1743–1794) had published *Traité Élémentaire de Chimie* (*Elementary Treatise of Chemistry*) in 1789. It was the first modern chemical textbook and presented a unified view of new theories of chemistry, contained a reinforcement of the law of conservation of mass, and repudiated the concept of "phlogiston." The latter was a theory that accepted the existence of a fire-like element called phlogiston that was contained within combustible bodies, which when released during combustion caused processes such as the rusting of metals, now understood to be oxidation. Lavoisier's textbook contained a list of elements that could not be broken down further, including oxygen, nitrogen, hydrogen, phosphorus, mercury, zinc, and sulfur. In his work, Lavoisier underscored the importance of experimentation rather than supposition. He wrote, "I have tried… to arrive at the truth by linking up facts; to suppress as much as possible the use of reasoning (sic. guess work), which is often an unreliable instrument which deceives us, in order to follow as much as possible the torch of observation and of experiment." Unfortunately, he was a victim of the early irrationality and brutality of the French Revolution (the Reign of Terror) and died on the guillotine because of his aristocratic heritage. When he requested a little time to complete some scientific experiments, the presiding judge purportedly responded, "The Republic needs neither scientists nor chemists; the course of justice cannot be delayed." Joseph Louis Lagrange (1736–1813), the most noted mathematician of that time upon hearing news of his friends execution remarked, "It took them an instant to cut off that head but France may not produce another like it for a century". Another giant of the day, Pierre-Simon Laplace (1749–1827) was advancing and revolutionizing mathematics, physics, and astronomy. His five-volume *Mécanique Céleste* (*Celestial Mechanics*) was monumental because it translated the geometrical study of mechanics, used by Isaac Newton, to one based on the calculus. Laplace also embraced probability theory in his treatise *Essai Philosophique sur les Probabilités* (Philosophical Essay on Probabilities) published in 1814. These thinkers exemplified the profound advances being made in science at that time.

In Britain, the aristocracy was losing its primacy, laws preventing child labor were to be enforced, and the institution of slavery was soon to be abolished in progressive countries. These changes fostered men of ingenuity, genius, and industriousness as opposed to members of the aristocracy, an essential element of progress. The entry of women as a force in science, with rare exception, awaited the more embracing atmosphere of the late twentieth century. Based on the previous observations and relatively crude inventions of many scientists (e.g., William Watson (1715–1787), Henry Cavendish (1731–1810), Charles-Augustin de Coulomb (1736–1806), Luigi Galvani (1737–1798), Alessandro Giuseppe Antonio Anastasio Volta (1745–1827), Georg Simon Ohm (1789–1854), and others) and incremental levels of understanding, Michael Faraday (1791–1867) devised an electric motor by 1821. Electricity would transform the way people lived and worked. The markedly improved steam engine devised by James Watt (1736–1819) and new means of transportation by land and sea, using steam energy, and its industrial applications would alter industry, commerce, and science and accelerate the industrial revolution that began in the early eighteenth century. The stethoscope and compound microscope would be applied to clinical medicine and pathology in new and dramatic ways and clinical and pathological medical findings would be integrated so as to advance our understanding of human disease.

Perhaps the most propitious of all the events of the time was Charles Darwin's (1809–1882) 5-year voyage on His Majesty's Ship Beagle in December 1831. This journey and his findings and their interpretation were to be the most significant scientific event of the nineteenth century and would provide a theoretical framework for all the biological sciences. One year after his return, in 1837, Alexandrina Victoria (1819–1901) would ascend to the throne of England at age 18 years and with her first cousin, husband and consort, His Royal Highness Prince Albert (Francis Albert Augustus Charles Emmanuel) (1819–1861), Duke of Saxony and Prince of Saxe-Coburg and Gotha, would oversee an era of profound artistic, industrial, and scientific advancement, and dramatic expansion of the British Empire, the Victorian Age, which encompassed her reign of 63 years and 7 months. During this period, the last 40 years of which followed Prince Albert's death, she appropriated an additional title, Empress of India.

An example of the broadening interest in science in the population at large was the enormous success of Arthur Conan Doyle's (1859–1930) fictional detective Sherlock Holmes, introduced in 1887 in "*A Study in Scarlet"* in which the magnifying glass was first used as an investigative tool in detective fiction. The character based on Joseph Bell (1837–1911), Conan Doyle's physician mentor at the Edinburgh Royal Infirmary, featured the skills of deductive reasoning and the ability to accumulate obscure clues and weave them into the fabric of enlightenment. His use of forensics, including analytical chemistry, toxicology, ballistics, and extraordinary skills of observation, reflected not only the advancement in science of that period but the acceptance of the importance of deductive reasoning, logic, and scientific methods. Indeed, when Conan Doyle, tired of his creation, killed him by sending him over Reichenbach Falls in the Swiss Alps with his adversary and archenemy, professor of

mathematics James Moriarty, the popular uproar of unhappiness was such that he later had to restore Holmes to his fictional life.

During this period, Claude Bernard (1813–1878), working at the Sorbonne, fostered the importance of the scientific method to ensure the objectivity of scientific conclusions in addition to making numerous advances in understanding human physiology, including the concept of homeostasis in the "milieu interieur." In 1865, he published *Introduction à l' Étude de la Médecine Expérimentale* (*An Introduction to the Study of Experimental Medicine*), a brilliant and insightful treatise on the objective approach to scientific inquiry. It was in this setting that medicine on the European Continent and in Britain was to see unprecedented progress and at which time the great universities and medical centers had been freed to follow new understandings in anatomy, physiology, and the embryonic discipline of biochemistry and to eschew dogma.

Hodgkin Lymphoma (Né Hodgkin's Disease)[2]

Evolution of Diagnostic Criteria

In 1832, a few months after Darwin set sail on the Beagle, a paper entitled *On Some Morbid Appearances of the Absorbent Glands and Spleen* was read to the Medico-Chirurgical Society of London by its Secretary, Robert Lee (1793–1877). In it, Thomas Hodgkin (1798–1866) described the clinical histories and gross postmortem findings of seven patients in whom the disease coupled prominent lymphadenopathy with splenomegaly [13]. Among the seven cases, one had been described by Robert Carswell (1793–1857), a Scottish physician and a gifted illustrator of medical pathology, who at age 35 years was named chair of pathology at London University. He was the first to illustrate the brain (pons) and spinal cord in multiple sclerosis [14]. He spent long periods of time in hospitals and mortuaries of Lyon and Paris painting watercolors and making pen and ink drawings of patients and postmortem specimens, completing 1034 paintings [15]. His atlas of brilliant watercolors, *Pathological Anatomy; Illustrations of the Elementary Forms of Disease*, was published in 1838, made possible by the invention of lithography in Germany 48 years earlier [16]. Much later, William Osler (1849–1919) remarked, "These illustrations have, for artistic merit and for fidelity, never been surpassed, while the matter represents the highest point which the science of morbid anatomy had reached before the introduction of the microscope" [17]. Carswell's images of cervico-clavicular-axillary lymphadenopathy, splenic involvement of the white pulp of the spleen, and perihepatic lymphadenopathy, among others, struck Hodgkin as an excellent representation of the disorder he was describing in his paper; he, therefore, included Carswell's case as the seventh in his report (Fig. 37.1). Unfortunately, journal publication techniques did not permit reproduction of Carswell's color paintings in Hodgkin's paper at that time.

At least two reports antedated Hodgkin's seven cases. David Craigie (1793–1866) in his text, *Elements of General and Pathological Anatomy* (1828), described a case with gradually enlarging glands, becoming hard and probably cancerous [18]. Craigie also cites a previous case reported in 1786 by William Cumberland Cruickshank (1745–1800) "in which the tracheobronchial lymphatic glands were affected with this morbid change to such an extent as to cause fatal suffocation." Cruikshank was an assistant to William Hunter in the latter's school of anatomy in London and was a gifted chemist as well as anatomist, first using chlorine for water purification and identifying carbon monoxide as being composed of carbon and oxygen, among other contributions. It was Hodgkin, however, who appreciated and articulated in his paper that these cases were a primary disease arising in the absorbent glands (lymph nodes) and not a process reacting to another "irritation." Although in retrospect several of Hodgkin's six cases may have represented different diseases (lymphoma of another type and infection), his paper was important because it was the first to call attention to diseases marked by lymphadenopathy associated with splenic enlargement in which it was proposed that the origin was in the lymphatic organs themselves, not secondary to another disorder [19].

Hodgkin was a remarkable man and in some ways his linkage with the earliest description of malignancies of lymphatic tissue has obscured his other contributions [20–22]. He was raised as a Quaker and wrote an essay at age 21 on the mistreatment of indigenous peoples, including the American Indians, by European colonists ("*Essay on the promotion of civilization*"). He was a physician's pupil at Guy's Hospital Medical School and attended the University of Edinburgh Medical School. He wrote his Doctor of Medicine thesis in Latin on the absorptive function of blood and lymph. This early interest in blood and lymph probably accounted for his focusing on lymphadenopathy in his seminal report. During Hodgkin's travels to France in 1821 he met René Laennec (1781–1826) who taught him how to use the stethoscope he had developed and, on his return to Guy's Hospital, he promoted the use of the stethoscope among

[2]For historical purposes, I have chosen to use the term Hodgkin's disease instead of Hodgkin lymphoma through most of this chapter. It took about 150 years after Wilks paper [25], in which he named the disease for Hodgkin, to establish the lymphocytic origin of the giant multinucleated cells in Hodgkin's disease and their clonal nature and, thus, through most of the time period discussed in this chapter Hodgkin's disease (later Hodgkin lymphoma) was the term used. The apostrophe "s" was deleted from eponymous medical designations in the mid-1980s (Editorial. Eponyms. *Ann Intern Med.* 1988;108:630–631.).

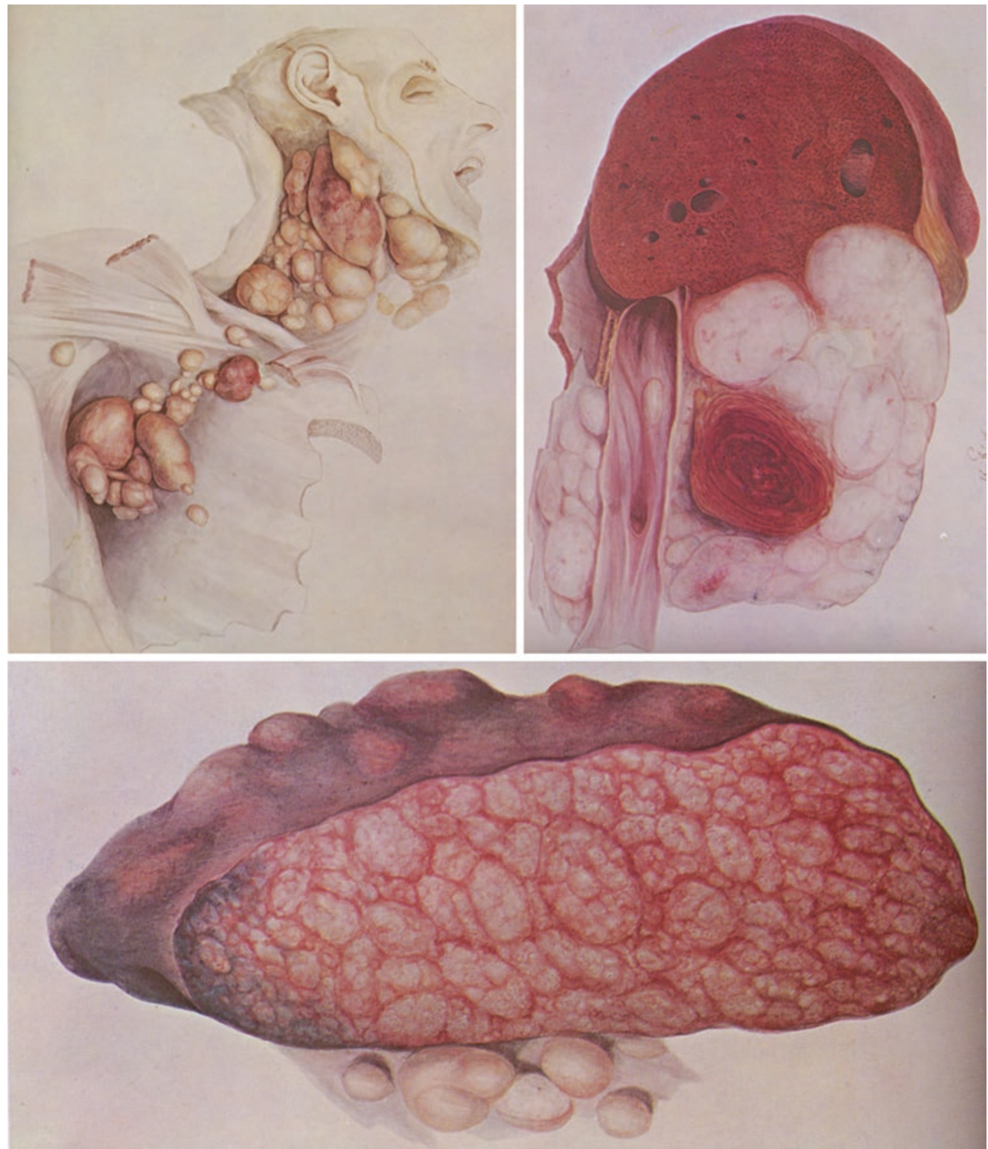

Fig. 37.1 Reproduction of Robert Carswell's vivid watercolor renditions of a patient he saw with lymphadenopathy and splenomegaly at the Hôpital Saint Louis during his travels to France [16, 17]. This patient was the seventh case used by Thomas Hodgkin in his paper of 1832 [13]. The patient was aged about 35 years with enlarged bilateral submandibular, cervical, axillary, and inguinal lymphatic glands ranging between a pea to a hen's egg in size according to Carswell. The drawings of the postmortem appearance of the cervico-clavicular-axillary lymph nodes, the perihepatic area, and the spleen are shown. The lymphatic glands under the liver surrounded the duodenum and "the great vessels of these parts." The tumor surrounded the aorta and vena cava, according to Carswell's description (These images were made available for reproduction through the generosity of University College Hospital and have been published previously in Dawson P. The original illustrations of Hodgkin's disease. Ann Diagn Pathol. 1999;3:386–89. Reprinted with permission of Elsevier)

British physicians. He became the curator of the pathological museum at Guy's Hospital and was one of the triumvirate of "the great men of Guy's," along with Richard Bright (1789–1858), a pioneering investigator of renal disease, and Thomas Addison (1793–1860), who described adrenal insufficiency in his classic monograph, *On the Constitutional and Local Effects of Diseases of the Suprarenal Capsules*. Although credited with the description of (Addisonian) pernicious anemia, the evidence is scant for this claim, as the anemia he described had few, if any, of the features expected. Several cases of lymphadenopathy Hodgkin used in his paper were patients under the care of Bright or Addison. Hodgkin had described aortic valve incompetence in a report entitled "On the retroversion of the valves of the aorta" published in 1829, 5 years before the Irish physician Dominic John Corrigan (1802–1880). Hodgkin described the nature and complications of appendiceal perforation 50 years before Fritz. Reginald Heber Fritz (1843–1913) was a pathologist at the Harvard Medical School who described the pathology of the appendix in appendicitis. Hodgkin was also interested in medical curricular innovations, arguing to increase science training for undergraduate medical students, and he was one of the earliest to propose a residency for physicians to improve postgraduate medical education (*An essay on medical education, 1828*).

Hodgkin was a proponent of analyzing clinical findings in the context of postmortem studies and he was both a skilled physician and the leading morbid anatomist of the day in Britain. Within 4 years of becoming museum curator, he had catalogued both the clinical and postmortem findings in 1600 specimens, arranged to show examples of the pathology of disease and their clinical correlations. He might be considered the father of the clinical–pathological conference, still highlighted today in the *New England*

Journal of Medicine. Because microscopy in the study of tissue was rudimentary at this time and infrequently used, only gross pathology was described. Richard Bright, Hodgkin's colleague at Guy's Hospital, reinforced Hodgkin's findings by including two of Hodgkin's cases in a detailed paper on abdominal tumors that he published in 1838, crediting Hodgkin with highlighting the malignant character and local origin of this disease of the absorbent glands, the term used for lymph nodes at the time. To his great disappointment, Hodgkin failed to be named to the position of Physician to Guy's Hospital after Bright's retirement. He devoted the later years of his life to humanitarian interests. He was a lifelong friend of and physician to Moses Hiam Montefiore (1784–1885), the renowned English financier, humanist, and, later, philanthropist. Montefiore was awarded a baronetcy by Queen Victoria. He was also briefly the Sheriff of London. Montefiore was deeply interested in rejuvenating the Jewish population of Palestine, then within the Ottoman empire. Hodgkin, as a Quaker, and Montefiore shared concern for the enslaved, dispossessed, and downtrodden of the world. Hodgkin was a strong opponent of the African slave trade and other inhuman behavior of the day. Montefiore shared these views and made major financial contributions to altering inhuman practices. Hodgkin died of a severe diarrheal disease while accompanying Montefiore on a trip to Palestine, the last of several they took together to Asia, Africa, and Europe, and is buried in what was at the time the Protestant cemetery in Jaffa. Montefiore erected a granite obelisk at his burial site on which he engraved a commeration to their 40 year friendship. Hodgkin's wife and brother included an inscription on the opposite side of the obelisk that included the phrase "*Humani nihil a se alienum putabit*" ("nothing human was foreign to him") [23, 24] (Fig. 37.2).

Fig. 37.2 Thomas Hodgkin was buried in the Protestant cemetery in Jaffa, Palestine, in 1866. He died of dysentery while on a trip with his close friend, Moses Montefiore. The inscription at the base of the obelisk that was erected by Montefiore, barely visible in this image, reads, "Here rests the body of Thomas Hodgkin, M.D., of Bedford Square, London, a man distinguished alike for scientific attainments, medical skill, and self-sacrificing philanthropy, died at Jaffa, the 4th day of April 1866, in the 68th year of his age, in the faith and hope of the Gospel. This epitaph is inscribed by his deeply sorrowing widow and brother to record their irreparable loss." They included in Latin "*Humani nihil a se alienum putabit*" ("nothing human was foreign to him"). On the reverse side of the obelisk, Montefiore's tribute is inscribed. A large blue plaque is affixed to the brick building in Bedford Square, London, that identifies it as Hodgkin's former home (This image of the obelisk was reproduced from http://en.wikipedia.org/wiki/Thomas_Hodgkin; image taken by Dr. Avishai Teicher)

Hodgkin's observations on diseases of lymphadenopathy with splenomegaly otherwise lay dormant for about 25 years, when in 1856, Samuel Wilks (1824–1911), independently, described ten cases in which he included four of Hodgkin's original cases [25]. The title "Cases of Lardaceous Disease and some allied affections" was principally related to cases of amyloidosis, which was the first such description of that disease. He separated the cases into four types and introduced a fifth type, which was exemplified by "a peculiar enlargement of the lymphatic glands frequently associated with disease of the spleen," which were four of Hodgkin's cases. Wilks thought at the time that his observations were original and hailed them as such. In his further research, Wilks uncovered Richard Bright's paper on abdominal tumors, in which Bright had included several of Hodgkin's case descriptions. Wilkes realized his error of omission and very generously made amends for his oversight by using the appellation, "Hodgkin's disease" in his paper "Cases with enlargement of the lymphatic glands and spleen (or Hodgkin's Disease)" in a subsequent series of 15 cases published in 1865 [26]. This eponymous designation has become ensconced in the medical lexicon.

In 1926, Herbert Fox (1880–1942), professor of pathology and director of the William Pepper Laboratory of Clinical Medicine at the University of Pennsylvania, traveled to Guy's Hospital and examined sections stained with hematoxylin and eosin prepared from the gross specimens of three of Hodgkin's six cases (Case II, IV, and VI), preserved in the Gordon Museum of Guy's Hospital in London [27]. The preservation of tissue in fixative was sufficient approximately 100 years later to permit Fox to confirm the histopathological diagnosis of Hodgkin's disease in two of the three cases and lymphoma of another type in the third. Fox was a distinguished bacteriologist and expert on typhoid fever and comparative bacteriology. He also had an interest in diseases of the blood and lymphoid organs. Seventy-three years later, in 1999, Fox's conclusions were supported by Robert (Robin) Nigel Poston (b.1944) in the Department of Experimental Pathology, King's College

and Guy's Hospital, London, who found Reed–Sternberg cells in the same two cases shown by Fox to be Hodgkin's disease on histological examination. He also established the multinucleated giant cells to be positive for Leu-M1 (CD15) using immunocytochemistry. Poston commented on the remarkable fact that this carbohydrate antigen survived the 160 years storage. The third case was compatible with another type of lymphoma in agreement with Fox's findings [28].

Hodgkin and his colleagues could not distinguish the various types of lymphoma and terms like primary lymphadenopathy or Hodgkin's disease in England, l'adenie or l'adenopathie in France, and pseudoleukämie in Germany, among others, were used to refer to what may have been Hodgkin's disease by today's understanding or various other types of lymphomas (malignant lymphadenopathy).

By the late nineteenth century, clinicians and pathologists had begun to define the histopathology of Hodgkin's disease more explicitly. The pathologists Paul Langerhans (1872), Auguste Ollivier and Louis-Antoine Ranvier (1876), William Smith Greenfield (1878), and William Richard Gowers (1879) had each described giant multinucleated cells, with multiple nuclei of large size, and distinct nucleoli in the lymph node sections of patients with a disease characterized by lymphadenopathy and often splenomegaly without abnormal cells in the blood [19]. The polychrome stains of Paul Ehrlich (1854–1915) had become available at the end of the nineteenth century and were used to describe the acidophilia of the nucleolus in the giant cells of Hodgkin's disease and the frequency of eosinophils in the lesion by Goldman in 1892 [29]. Greenfield was the first to publish drawings of the giant cells characteristic of the pathological appearance of Hodgkin's disease [30]. At a symposium on diseases of the lymphatic system, including lymphadenoma and leukemia, chaired by Wilks in 1878, vivid illustrations of lymphadenopathy were presented [30, 31]. At that symposium, Greenfield presented drawings of the pathology of lymph node sections in several cases of Hodgkin's disease showing their similarity and the large number of multinucleated cell that characterized the lesions. His description of the lymph node section in a case of lymphadenoma (another synonym for Hodgkin's disease at the time) read, "… a large number of multinucleated cells adherent to trabeculae, well seen on washing away the lymph cells. These multinucleated cells, containing from four to eight or twelve nuclei were often collected in clusters in the parts of the gland especially where the fibrous change was progressing (see Pl. XIII, Fig. 37.2)." He emphasized the distinction between metastatic carcinoma involving lymph nodes and lymphatic cancers originating in the nodes. He discussed lucidly the clinical basis with which to distinguish lymphadenoma from lymphatic leukemia. He also highlighted the characteristic contiguous spread of Hodgkin's disease to neighboring lymph nodes [30].

Paul Langerhans (1847–1888) [32] and Julius Dreschfeld (1845–1907) [33] further defined the breadth of the clinical and pathological presentations of Hodgkin's disease, a forerunner to modern classifications (Table 37.1). Langerhans

Table 37.1 Chronology of major advances in the histopathological classification, anatomical distribution (stage), and tissue of derivation of Hodgkin lymphoma: historical aspects

Year	Event	References
1832	Lymphadenopathy and splenomegaly considered a disease intrinsic to lymphatic tissue and not a reactive process	Hodgkin [13]
1838	Color paintings of the gross anatomical findings in the lymph node chains, spleen, and perihepatic nodes in Hodgkin's disease	Carswell [15, 16]
1856	Cases previously reported by Hodgkin's rediscovered	Wilks [25]
1865	More cases of lymphadenopathy and splenomegaly reported and Hodgkin given priority by calling the cases "Hodgkin's disease"	Wilks [26]
1870–1879	Multiple observers, notably Greenfield, describe and illustrate the multinucleated giant cells characteristic of the disease	Greenfield [30]
1892	Hodgkin's disease (syn. pseudoleucocythemia) distinguished from lymphosarcoma and classification of acute and chronic Hodgkin's disease proposed	Dreschfeld [33]
1898	Histological picture of Hodgkin's disease characterized, including diagnostic giant cells and mononuclear variant cells. Concludes disease caused by the tubercle bacillus because of striking coincidence of its presence in patients with Hodgkin's disease	Sternberg [34]
1902	Histological pattern characterized with elegant drawings of the appearance of lymph node lesions, including giant multinuclear cells. Concludes that it is a distinctive disease	Reed [35]
1947	Hodgkin's disease divided into three subtypes: paragranuloma, granuloma, and sarcoma	Jackson and Parker [36]
1950	Importance of clinical stage in the outcome of radiotherapy and a proposed system introduced that formed the basis for future staging classifications	Peters [37]
1952	Lower-extremity lymphangiography for visualization of pelvic and retroperitoneal lymph nodes	Kinmonth [38]
1966	Hodgkin's disease reclassified into six histopathological types	Lukes et al. [39]
1966	Rye conference arrives at consensus of simplifying classification into four histopathological types of Hodgkin's disease: lymphocyte predominance (15%), nodular sclerosis (40%), mixed cellularity (35%), lymphocyte depletion (10%)	Lukes et al. [40]

(continued)

Table 37.1 (continued)

Year	Event	References
1966	Rye proposal to stage the disease into four categories based on anatomical distribution and to split each into an (A) and (B) based on the absence or presence of constitutional symptoms (fever, night sweats, or pruritis)	Rosenberg [41]
1969	Laparotomy, abdominal lymph node biopsy, and splenectomy for staging of Hodgkin's disease	Glatstein et al. [42]
1971	Revised four-part Ann Arbor staging system for clinical and pathological staging of Hodgkin's disease	Carbone et al. [43]
1987	Identification of Epstein–Barr virus and its DNA in Hodgkin tissue and in Reed–Sternberg cells in about 40% of cases	Weiss et al. [44, 45]
1994	Identification of Reed–Sternberg cells as a clone derived from a germinal center B lymphocyte, conclusively designating Hodgkin's disease a B-cell lymphoma (Hodgkin lymphoma)	Küppers et al. [46–48]

was a distinguished anatomic pathologist who applied newer staining techniques to study tissue sections. He had worked with Virchow and Julius Cohnheim (1839–1884) in Berlin, where he finished his medical studies. He is most famous for his detailed description of the histological anatomy of the pancreas and the identification of the pancreatic islets (of Langerhans). He also identified a specific cell in the dermis, the Langerhans cell, but at the time was uncertain of its function and wondered if it was of neural origin because of its shape. He also identified phagocytic cells distributed in various organs, such as liver, spleen, and marrow, by their ingestion of cinnabar injected into the circulation. These were the scattered parts of the mononuclear phagocyte system, although that concept took some time to evolve after Aschoff dubbed it "the reticuloendothelial system," a system which in fact did not involve directly either reticular or endothelial cells, as we would learn subsequently. His conceptualization of a distributed system devoted to phagocytosis of foreign particles was important, however, regardless of the misnomer.

In 1892, Dreschfeld described what he termed "acute" and "chronic" Hodgkin disease [33]. He presented detailed descriptions of cases with cervical and mediastinal disease or abdominal disease to which he applied the designation typhoidal Hodgkin's disease because its manifestations simulated typhoid fever. He commented on the occasional high fever with intervals of nearly normal temperature, by that time already referred to as the fever pattern of Pel and Ebstein. Pieter Klazes Pel (1852–1919), a physician in Amsterdam, first reported this pattern of fever in a case of pseudoleukämie, a synonym used in continental Europe for lymphomatous diseases including Hodgkin's disease [49], 2 years before Wilhelm Ebstein's (1836–1912) description. Ebstein was a distinguished German academic physician and a world's authority on nutrition. Pel felt the fever was a manifestation of pseudoleukämie and Ebstein thought it represented a separate condition. Dreschfeld's descriptions of types of Hodgkin's disease were hampered by the inability of drawing histopathological distinctions among the various types of malignant diseases of lymph nodes. Dreschfeld was the most distinguished physician in North England in the late nineteenth century. Although born in Bavaria, he immigrated to Manchester, where he excelled in chemistry and mathematics and entered the Royal School of Medicine in Manchester. He continued medical studies in Würzburg at a time when the latter school was among the great medical schools in Europe with a distinguished faculty that included Heinrich von Bamberger (1822–1888), Virchow, Albert von Koelliker (1817–1905), and others. He became a skilled pathologist, microbiologist, practicing physician, and a renowned scholar. Upon his death, a biography of his life was published as a tribute by his admiring colleagues, which included his published papers, several seminal, and described his remarkable achievements in medicine [50].

Detailed histopathological descriptions of the large component of inflammatory cells, the significance of eosinophilia in some cases, and giant cells that were bi- or multinucleated with large nucleoli had by this time been published, as noted earlier. Dreschfeld and others considered the disease infectious, even though the term malignant was often applied.

In 1898, Carl Sternberg (1872–1935) in Vienna [34] and, shortly thereafter, Dorothy Reed in 1902 [35], working at John's Hopkins University in Baltimore, were credited with the first definitive and thorough histopathological descriptions of the disease and its characteristic, diagnostic giant cells, thereafter dubbed Sternberg–Reed or Reed–Sternberg cells, depending on the priority given to the earlier date of the description (Sternberg) or the more detailed drawing of the cells and the more astute description of their pathological significance (Reed). Sternberg not only carefully described the Sternberg–Reed cell but its mononuclear variant. He wrote, "There are also large pale eosinophilic protoplasmic bodies with a pale eosinophilic, large nucleus, structures corresponding to the large cells." Most of Sternberg's cases had evidence of tuberculosis and he became convinced that Hodgkin's disease was inflammatory, a reaction to infection with the tubercle bacillus. He considered his cases as tuberculosis masquerading as pseudoleukämie. He later modified his views slightly, forced to retreat by the cases of Hodgkin's disease in which careful search could not find evidence of the tubercle bacillus.

Dorothy Mabel Reed Mendenhall (1874–1964) did her undergraduate work at Smith College and additional premedical

studies at the Massachusetts Institute of Technology and became one of the few women up to that time to graduate from Johns Hopkins Medical School and intern at Johns Hopkins Hospital, where she served under William Osler. She, thereafter, became a Pathology fellow under the direction of William Henry Welch (1850–1934). From 1893, Welch directed the rise of Johns Hopkins University School of Medicine, where he started the country's first university department of pathology. He recruited William Osler, the first physician-in-chief, and William Stewart Halsted (1852–1922), the first chief of surgery, to the faculty and was the medical school's first dean. His curriculum revolutionized medicine in the United States by demanding that students study physical sciences and be actively involved in clinical duties and laboratory work. Among Reed's interests in pathology was the histological nature of Hodgkin's disease. Reed recognized Hodgkin's disease as an independent disorder and not a form of tuberculosis. She stated in her paper in 1902 (2 years after graduation from medical school) "… from the descriptions in the literature and the findings in eight cases examined, that Hodgkin's disease has a peculiar and typical histological picture and could rightly be considered a histopathological disease entity" [35]. Dorothy Reed according to autobiographical writings was not given the opportunity to advance her position at Hopkins because of her gender. She decided that she must leave and Welch arranged for her to work at the new Babies Hospital affiliated with the medical school at Columbia University in New York City. After a short interim residency position in New York City, she was accepted into the internship in pediatrics at the Babies Hospital. She worked under the direction of Luther Emmet Holt, Sr. (1855–1924), a pioneer in pediatrics, author of the first major textbook on the subject and author of "The care and feeding of children." Dorothy Reed left medicine to marry Charles Mendenhall who became Chair of Physics at the University of Wisconsin. She had four children, two of whom died in the first year of life. Her son, John "Blackjack" Mendenhall, became a prominent thoracic surgeon and another son, Thomas C. Mendenhall, was a professor of History at Yale University, and served as the sixth President of Smith College, Reed's alma mater. Dorothy Mabel Reed Mendenhall died of heart disease in 1964, at the age of 89. One can only speculate as to the contributions she would have made had she been given the opportunity to continue her work as an experimental pathologist.

Recognizing Histopathological Heterogeneity

As the histopathology of Hodgkin's disease was subject to more understanding and careful assessment, it was evident that the disease had several distinctive histological patterns. James Stephen Ewing (1866–1943), the first professor of pathology at Cornell University Medical College, in his classic textbook, *Neoplastic Disease; A Text-Book on Tumors*, published in 1919, describes the typical features of what he called "Hodgkin's granuloma" and, also, a clinically progressive disease in which the lymph node biopsy had pleomorphic variations of Reed–Sternberg cells to which he applied the designation, "Hodgkin's sarcoma" [51]. The disease was still thought to be the tissue manifestation of tuberculosis although cases in which the tubercle bacillus was not evident raised doubts in the minds of some observers. Sternberg still held to the idea that tuberculosis was the major cause; he conceded that there may be others. The idea that "tuberculosis follows Hodgkin's disease like a shadow," commented on in Ewing's chapter, was misinterpreted as the tubercle bacillus being an etiological agent rather than a secondary infection in a susceptible host. Eventually studies showed that only about one in four patients with Hodgkin's disease had coincidental tuberculosis and Hodgkin's disease was rare in patients with tuberculosis. Many prominent pathologists argued against the causal relationship and the concept withered.

In the mid-1940s, Henry Jackson, Jr. (1892–1968) and Frederic Parker, Jr. (1890–1969), at the Thorndike Memorial Laboratory and the Mallory Institute of Pathology at the Boston City Hospital and Harvard Medical School, built on earlier concepts and proposed three subtypes of Hodgkin's disease based on clinical presentation and histopathology: paragranuloma (small nodes in the neck, usually, and Reed–Sternberg cells in the pathological specimen), granuloma (much higher frequency of abdominal adenopathy, more effacement of nodes, more atypia to Reed–Sternberg cells), and sarcoma (notably retroperitoneal nodes, nodal confluence, invasion of neighboring intestinal structures, Reed–Sternberg cells, and many variants of such cells) [36]. The categories signified on average more rapidly progressive disease from paragranuloma to sarcoma. The classification suffered by the fact that about 90% of cases fell into the granuloma category and the histopathology of the cases classified as granuloma was very diverse, not uniform.

First, Robert J. Lukes (1922–1995) in 1963 and then Lukes, James J. Butler (d. 2004), and Ethel B. Hicks in 1966 presented a classification that provided six histopathological subtypes of Hodgkin disease, which addressed the criticism of the tripartite classification of Jackson and Parker and provided a distribution of cases among the six categories that was more discriminating. The distinctions were confirmed by a median survival that ranged from 12 to 2 years in parallel with advancing severity of the six categories [39]. At the time of this work, Robert Lukes was professor of pathology at the University of Southern California School of Medicine and chief of hematopathology at the Los Angeles County Hospital. Butler had collaborated with Lukes when both were at the Armed Forces

Institute of Pathology in Washington, D.C. where Lukes had been chief of hematopathology. Thereafter, Butler went to the M.D. Anderson Cancer Center in Houston, Texas. Lukes and Butler were leading authorities on the histopathology and classification of Hodgkin's disease and other lymphomas. Ethel Hicks was a statistician at the Armed Forces Institute of Pathology collaborating with Lukes and Butler on the analysis of their data. The six histopathological categories, although considered useful in the hands of experts, were difficult to reproduce by pathologists in various centers around the world and their proposal was modified in 1965 at a conference held in Rye, New York, in which the six histopathological subtypes were collapsed into four subtypes: lymphocyte-predominant, nodular sclerosis, mixed cellularity, and lymphocyte-depleted Hodgkin's disease [40]. Several modifications have superseded the Rye classification and the most current classification recommended by the World Health Organization panel on tumors of hematopoietic and lymphoid tissues has five categories of Hodgkin lymphoma, judged histopathologically [52].

Importance of Stage of Disease

In addition to histopathological classification of Hodgkin's disease, the distribution (stage) of the disease was determined to be important in assessing therapeutic approaches. Mildred Vera Peters (1911–1993), a Canadian radiotherapist, and a pioneer in the treatment of the disease with radiotherapy, described a clinical staging system in 1950, emphasizing the diagnostic evaluation of the anatomic extent of disease [37]. She proposed three stages depending on extent of disease and recognized the additional significance of constitutional symptoms of fever, night sweats, and weight loss. This proposal, describing three stages of the disease, formed the basis for future modifications, which split Peters' third stage into a third and fourth stage. In 1952, John Bernard Kinmonth (1916–1982), working at St. Bartholomew's Hospital, London, introduced lower-extremity lymphangiography that allowed visualization of the iliofemoral, pelvic, and retroperitoneal lymph nodes by imaging the size and pattern of injected contrast material in the lymphatic channels and nodes. This approach was more sensitive and specific than physical examination of the abdomen or other radiographic methods for these previously inaccessible regions [38]. Kinmonth had coauthored a textbook of vascular surgery and was both a pioneer and the leading surgical authority on lymphatic imaging. In 1972, he published his monograph entitled "*The Lymphatics*," which was updated in 1982 [53]. The frequency of unsuspected splenic involvement was appreciated when laparotomy with splenectomy and biopsy of splenic hilar, para-aortic and mesenteric lymph nodes, and liver was introduced for staging the disease [42]. These diagnostic procedures led to improved understanding of the pattern of dissemination of the disease and correlated well with prognosis, culminating in the advanced concepts of staging codified at the Rye conference in 1965 [41], and further refined at the Workshop on the Staging of Hodgkin's Disease in Ann Arbor, Michigan, in 1971 [43]. Although the introduction of computed tomography and positron emission tomography has superseded these invasive methods (lymphangiography and laparotomy), they were important at the time to correlate extent of disease with therapeutic approach and outcome.

The first commercially viable computed tomography scanner was invented by Godfrey Newbold Hounsfield (1919–2004), an engineer in Hayes, United Kingdom at EMI Central Research Laboratories using X-rays. Hounsfield conceived his idea in 1967 and it was publicly announced in 1972. Allan McLeod Cormack (1924–1988), a physicist, at Tufts University in Boston, Massachusetts, independently invented a similar process, and both Hounsfield and Cormack shared the 1979 Nobel Prize in Physiology or Medicine for this dramatic advance in diagnostic imaging by combining computer technology with X-ray technology. In May 1974, Siemens Medical Engineering introduced the first computed tomography system. This new technology was advanced 2 years later when Siemens marketed a computed tomography scanner that provided physicians with cross-sectional images of tumors. By 1977, Siemens had developed a system that integrated the radiation source and a detector system into a "gantry," a mechanism that permits the instrument to rotate around the patient to achieve whole-body computed tomography examinations. By 1985, it became possible to image anatomical features in three dimensions. The first spiral scanner was released in 1989, making it possible to scan a patient's lungs during a single breath-hold. Over the intervening decade and a half, computed tomography technology has been refined to the point that it can now visualize minute tumors, increasing diagnostic sensitivity.

In 1991, David Townsend (b.1945) and Ronald Nutt (b.1938) had what became known as "the epiphany in the Alps" in Geneva, Switzerland, when they first conceived coupling positron emission tomography (PET) with computerized axial tomography (CT). Townsend was on the faculty at the University of Geneva and at the Cantonal Hospital in Geneva from 1979 to 1993; he and an electrical engineer, Ronald Nutt, began exploring the idea of combining PET and CT to improve identification of human tumors. They were stimulated by a surgeon who indicated that each was helpful, but together they would be extraordinary. In 1993, the University of Pittsburgh hired Townsend to run the physics and instrumentation program of their PET facility. Nutt had started a company in Knoxville, Tennessee, and they continued collaborating on the combined PET-CT

device in the United States. Siemens launched a joint venture with Nutt's company to build the prototype instrument. Other manufacturers began developing versions of a combined scanner as well. One obstacle to use of PET-CT was the difficulty and cost of producing and transporting the radiopharmaceuticals used for PET imaging, which are usually short-lived. For instance, the half-life of radioactive fluoride used to trace glucose metabolism, using fluorodeoxyglucose (FDG) is 2 h. Its production requires a cyclotron as well as a production line for the radiopharmaceuticals. PET-CT has revolutionized many fields of medical diagnosis by adding precision of anatomic localization to functional imaging. This duality enhances cancer staging, surgical planning, radiation therapy, and the assessment of therapeutic results. Although the combined device is considerably more expensive, it has the advantage of providing both functions as stand-alone examinations, being, in fact, two devices in one.

The Cell of Origin and the Presence of Epstein–Barr Virus

Hodgkin suspected that the syndrome (disease) to which he was calling attention, lymphadenopathy, often with splenomegaly, without an apparent infectious cause was a disease intrinsic to lymphatic tissue. Bright and Wilkes reinforced that concept. At the beginning of the twentieth century, Hodgkin's disease was considered a neoplasm by some experts (e.g., James Ewing).

For most of the twentieth century, debate raged as to whether a lymphocyte or a macrophage was the closest analogue to the tumor cell. The proposal that some forms of Hodgkin's disease (slowly progressive in young persons) may be infectious persisted, especially for the first 65 years of the twentieth century. In part, this confusion was related to the inability to identify a clonal population of neoplastic cells in the various histopathological subtypes of the disease. The latter problem was related in large part to the extremely small proportion of clonal Reed–Sternberg cells and the large fraction of polyclonal lymphocytes, eosinophils, and macrophages in the lesion. Hodgkin's disease, like Langerhans cell histiocytosis, represents an example of a neoplastic transformation in which the tumor cells (monoclonal Reed–Sternberg or monoclonal Langerhans cells) are an extremely small proportion of the cellular infiltrate (<1%), a very unusual state for clonal (neoplastic) growths. The nature of the Reed–Sternberg cell remained a center of controversy until techniques became available to show that it has the phenotype and genotype of a monoclonal B lymphocyte and represented the neoplastic clone in all subtypes of Hodgkin's disease. Thus, Hodgkin disease could be renamed Hodgkin lymphoma with certainty. Ralf Küppers (b. 1962) and colleagues at the Institute for Genetics and Department of Pathology in Cologne developed techniques to isolate single Reed–Sternberg cells from frozen tissue sections of biopsies by micromanipulation and to do polymerase chain reaction testing on single cells. Their findings resulted in establishing that Reed–Sternberg cells had rearranged variable (V) region immunoglobulin genes and, thus, were derived from monoclonal B lymphocytes, since V gene rearrangements are restricted to B-lymphocytic lineage cells and are highly specific for a B-cell clone [46–48].

Hodgkin's disease was established as a neoplastic B-lymphocytic disorder with striking clinical, histopathological, and epidemiological heterogeneity. For this reason it has been debated as to whether it had several etiological initiators or was the variable host response to a single cause. An infectious inciting agent had been proposed since the nineteenth century during which time distinguished proponents of a role for *Mycobacterium tuberculosis* kept it in the forefront (Dreschfeld, Sternberg, and others). The presence of fever, reactive inflammatory cells dominating the lymph node infiltrate, a bimodal age incidence curve in Western societies, a younger age of incidence in a large proportion of affected persons than most neoplasms, and social-class-related relative risk had led some to consider it an unusual reaction to an infection in affected younger persons.

In 1987, Lawrence M. Weiss (b. 1956), currently Chair of Pathology at City of Hope, working with colleagues at Stanford University Medical Center reported the presence of Epstein–Barr virus DNA in the tissues of some patients with Hodgkin's disease [44]. Subsequently Epstein–Barr viral genomes [45] and viral messenger RNA [54] were identified in Reed–Sternberg cells. These findings have been replicated in several laboratories. Although Epstein–Barr virus sequences are found on average in four of ten patients, its relationship to the disease is found more intriguing if one looks more specifically at the association. The prevalence of Epstein–Barr virus is particularly notable in cases with either mixed cellularity or lymphocyte-depleted subtypes and is infrequent in nodular sclerosis or lymphocyte-rich classical Hodgkin disease. It is rarely present in nodular lymphocyte-predominant Hodgkin's disease. In Central and South American pediatric cases, the virus is present in over 90% of cases [55]. The significance of Epstein–Barr virus genomes in the tumor cells of patients with Hodgkin's lymphoma remains an intriguing association without as yet a full understanding of its role in the pathogenesis of the neoplasia. There are compelling findings, however, that an etiological role for the virus in cases in which it is found [55]. The Epstein–Barr virus is associated with three other diseases of B lymphocytes: infectious mononucleosis, endemic Burkitt lymphoma, and B-cell lymphoma in the setting of immunosuppression.

Therapy

Radiation Treatment

Wilhelm Conrad Röntgen (1845–1923) was a physicist with a background in mechanical engineering. While a professor of physics at the University of Würzburg, at which he had colleagues of the ilk of Hermann von Helmholtz and Ludvig Valentin Lorenz (1829–1891), he was studying the phenomenon of passing an electric current through a gas of very low pressure. His work was built on the discoveries of several predecessors, including William Crookes (1832–1919), Henrich Rudolph Hertz (1857–1894), and Eugen Goldstein (1850–1930), which had led to the development of the cathode ray tube. Röntgen's experiments on cathode rays led, however, to his discovery of a hitherto unknown ray form that he dubbed "X-rays," using the mathematical symbol "X" for an unknown quantity. When he exposed his wife's hand to these rays, while it was held over a photographic plate, he observed upon development of the plate the dark shadows of the bones of her hand and the ring on her finger with much lighter shadows from the outline of the skin of the hand. The first X-ray of human tissue had been made. Röntgen published his landmark paper on the discovery of a new type of electromagnetic ray, *Über eine neue Art von Strahlen* (On a New Kind of Ray), 50 days after his discovery, on 28 December 1895. The achievement resulted in his being named the first Nobel Prize Laureate in Physics in 1901, a very propitious start for the Nobel Foundation. Arguably, his discovery must be among only a handful that have had such an enormous and long-lasting impact on the understanding and diagnosis of human disease and an incalculable contribution to maintaining and restoring human health. Later, X-rays were shown to be of the same electromagnetic nature as light but vibrating at a much higher frequency.

In his will, Alfred Bernard Nobel (1833–1896) asked that the Prize be given to a discovery of benefit to mankind achieved in the previous year, a requirement to which the Nobel Foundation, wisely, did not adhere. Nonetheless, few if any Laureates made a discovery and contribution to medicine either recognized so quickly by the Nobel Foundation or of the magnitude of Röntgen. His discovery revolutionized the diagnosis and treatment of disease that continues today with the application of computed tomography and with advances in the instruments and techniques of radiotherapy.

By the first few years of the twentieth century, X-ray therapy was applied successfully to the treatment of Hodgkin's disease by William Allen Pusey (1865–1940) at the University of Illinois [56] and Nicholas Senn (1848–1908) at Rush Medical College in Chicago [57] (Fig. 37.3). In 1902, Pusey and, in 1903, Senn each published pretherapy and posttherapy images showing the disappearance of massive lymphadenopathy after X-radiation of the lymph nodes in patients with Hodgkin's disease. Pusey was a dermatologist in Chicago at the College of Physicians and Surgeons (later University of Illinois Medical School) who used radiotherapy for treatment of skin lesions and also tumors. He was the founder and first chief editor of the *Archives of Dermatology and Syphilology*. Senn, born in Switzerland, and chair of surgery

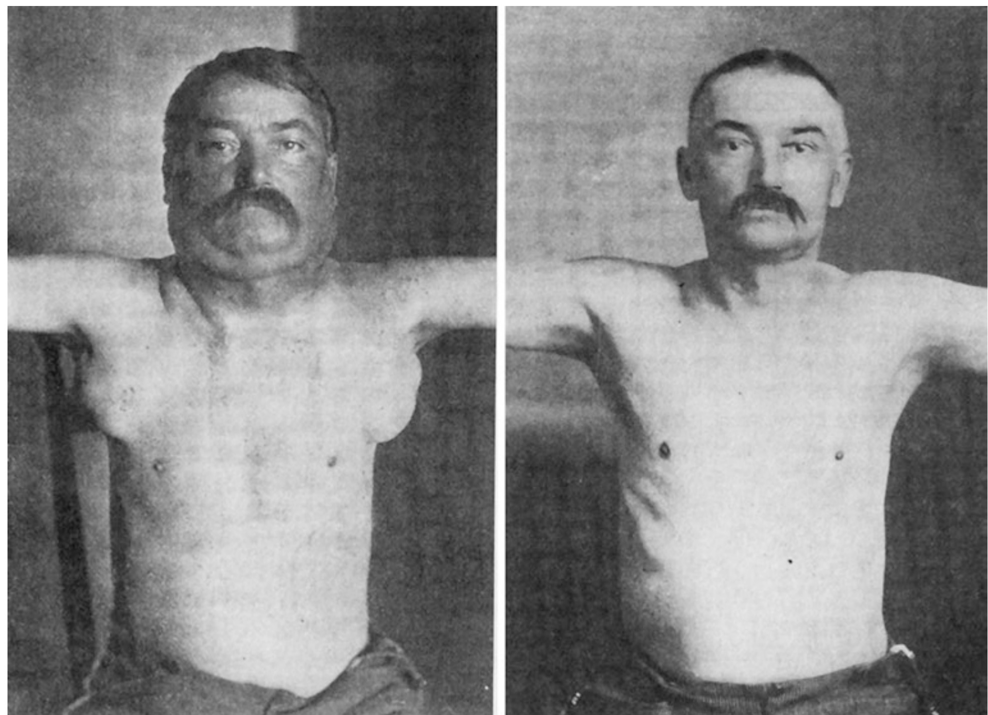

Fig. 37.3 An example of the salutary effects of early orthovoltage radiation therapy for Hodgkin's disease. *Left*, before radiation. *Right*, after radiation. Pusey in 1902 [55] and Senn in 1903 [56] reported the first use of radiotherapy, which dramatically shrank the lymph nodes of patients with Hodgkin's disease, at least transiently (Reproduced with permission from Senn N. Therapeutic value of Röntgen ray in treatment of pseudoleukemia. N Y Med J. 1903;77:665–68.)

at Rush Medical College in Chicago, was one of the most distinguished academic surgeons of his day. He authored numerous medical articles and several textbooks including *Principles of Surgery*. He was president of the American Medical Association. His fascinating life's story, travels, military service, bibliophilia, varied contributions to medicine, and other accomplishments are worthy of study for one interested in the history of medicine.

In 1896, stimulated by Röntgen's discovery, Antoine Henri Becquerel (1852–1908), working at the University of Paris, discovered that uranium salts emitted rays that resembled X-rays in their penetrating power. He demonstrated that this radiation did not depend on an external source of energy, but seemed to arise spontaneously from uranium itself. He was awarded the Nobel Prize in Physics in 1903 for this profound discovery. Energized by Becquerel's groundbreaking discovery, Marie Curie (Maria Salomea Sklodowska Curie) (1867–1934) and her husband, Pierre Curie (1859–1906), created the theory of radioactivity, developed techniques for isolating radioactive isotopes, and discovered two new elements, radium (named by Marie Curie for its "radioactivity") and polonium, the decay product of radium (named by Marie Curie for her country of birth). One gram of radium was found by the Curies to emit 3.7×10^{10} disintegrations per second, an amount of radioactivity since referred to as a Curie (Ci), named after Pierre Curie, as designated by his widow, Marie. In 1975 the General Conference on Weights and Measures made the unit the becquerel (Bq), equivalent to one reciprocal second, and gave it official status as the SI unit of activity. Thereafter, the Ci was defined as 3.7×10^{10} Bq or 37 GBq.

Soon after the discovery and purification of radium, it became commercially available from a company in Belgium. Marie Curie was the first to use it for cancer treatment. She became the first women professor at the University of Paris, the first women to be named a Nobel Prize Laureate (she shared the physics prize in 1903 with her husband and Becquerel), the first person to receive the Nobel Prize twice (the Chemistry Prize in 1911), and the first Laureate to have an offspring win the Nobel Prize. Her daughter was the second woman to be awarded the Nobel Prize in Chemistry a quarter of a century later. Irene Joliet-Curie, Marie Curie's daughter, and her son-in-law Frédéric Joliot shared the Nobel Prize in Chemistry in 1935. Marie Curie originally wanted to work in Poland after her studies in physics and mathematics were completed in Paris, but Kraków University denied her entry, ostensibly because she was a woman. Curie's first prize devolved from the conclusion that radiation did not depend on the arrangement of the atoms in a molecule; it must be linked to the interior of the atom itself. This discovery was revolutionary and conceptually was her most important contribution to the development of atomic physics. Chemists considered the discovery and isolation of radium the greatest event in chemistry since the discovery of oxygen. Her second prize devolved from the demonstration for the first time that an element could be transmuted into another element. This finding revolutionized chemistry and began a new epoch in the field.

The practical implications of the availability of X-ray machines and radium for therapy of cancer were soon evident in Europe and the United States. The establishment of a facility to study and treat cancer in Manchester, England, eventually developed into the Christie Hospital and Holt Radium Institute. It had begun using X-ray therapy in 1901 and radium treatments in 1905. In the United States, a Department of Cancer Surgery and Radiation Therapy was established at The New York Cancer Hospital (later the General Memorial Hospital for the Treatment of Cancer and Allied Diseases) in 1912 (see discussion later in this chapter). Surgeons saw radiation therapy as a treatment of cancer through its caustic effect, a dissolution instead of an excision.

Within a few years of Röntgen's primitive X-ray machine, more advanced diagnostic and therapeutic X-ray devices were being developed. The advances spawned by Röntgen's discovery have made an enormous contribution to the diagnosis and staging of lymphomas, using a series of imaging devices, each more sophisticated and useful than the last (e.g., from X-radiography to computed tomography to PET-CT). Pusey's and Senn's dramatic, but relatively short-lived, shrinkage of the lymph nodes in patients with Hodgkin's disease treated with X-ray therapy were the first efforts to bring a logical and effective approach to its treatment. Their X-ray equipment, using a Crookes' tube, generated beams of low energy and low intensity. One of the most significant advances in radiology and radiotherapy was developed by William D. Coolidge (1873–1975), Director of the General Electric Research Laboratory. His device, incorporating an extremely high vacuum, the use of a heated cathode as a source of electrons, and a tungsten anode, was revolutionary in radiological tube technology. The Coolidge tube described in 1913 produced X-rays generated at 140 kv and by 1922 a tube operating at 200 to 250 kv permitted "deep therapy." The 1920s through the 1950s was the era of kilovoltage radiation therapy [58].

Much debate existed on the best use of X-radiation for Hodgkin's disease; arguments about doses, fields, and treatment schedules raged during the first 30 years of the twentieth century. René Gilbert (1892–1962) working in Geneva, Switzerland, was a pioneer in the treatment of Hodgkin's disease with radiation. From the mid-1920s to the late 1930s, he pursued an approach that set the stage for the later advances in radiotherapy, which resulted in the prolonged survival for advanced disease and cure of more localized disease [59]. He was handicapped by having only relatively low voltage equipment, but he emphasized (1) formulating a treatment plan after careful assessment of the extent and location of the disease, (2) treating intensely as possible at the time of initial therapy by focusing on known sites of disease, and, thereafter (3) treating neighboring, apparently

uninvolved sites, as the patient's tolerance permitted, recognizing the likelihood of microscopic disease in those locations [60]. In 1931, he presented survival data for patients followed for nearly 10 years in some cases, extraordinary results for the time, given the limitation of the radiotherapeutic equipment of his day.

Charles Brigman Craft (1909–1979) in 1940, working at the University of Minnesota Hospital, published his more carefully designed comparison between untreated patients and those treated with radiation and confirmed, quantitatively, the impact of radiation therapy: untreated patients had a 5- and 10-year survival of 5.8 and 0% and radiotherapy-treated patients had a 5- and 10-year survival of 23.4 and 11%, respectively [61]. Indeed, since two patients were alive at 13 years after therapy, Craft's treatment may have resulted in the first cures of the disease. His publication in the proceedings of the staff meetings of the University of Minnesota Hospital did not gain wide attention.

Vera Peters was the first to report probable cures of Hodgkin's disease with radiation based on careful analysis of treated patients. Peters joined the faculty of the Radiotherapy Institute at the Toronto General Hospital working with its leader, Gordon Richards (1885–1949). Richards had installed a 400 kv radiotherapy machine and was using the principles espoused by Gilbert in his approach to therapy. In 1947, Richards suggested that Peters review their cases of Hodgkin's disease because he was impressed with the longevity of many of the patients he had treated [62]. She completed her review in 1949, but Richards had died in January of that year and never learned the results of the study he suggested. In her landmark paper, published in 1950, of 113 cases confirmed by histopathological examination to have Hodgkin's disease, she showed that the extent of the disease at the time of diagnosis was the most important correlate with survival, resulting in the Peters staging system commented on earlier [37]. She found that constitutional signs of fever, night sweats, and weight loss had a negative consequence on treatment outcome. The 10-year survival of patients classified by Peters as stage I was 79% and this resulted in a much more hopeful view of the treatment of the disease. Initially the phrase "curability of Hodgkin's disease" was met with disbelief. By the mid-1960s, the confirming work of others changed this attitude. Eric Craig Easson (1915–1983) and Marion Howard Russell (1907–1966) at the Holt Radium Institute and Christie Hospital in Manchester showed that the event-free survival at 15 and 20 years of their patients treated with radiation was little different from the rates at 10 years, implying that these patients were cured of their disease, as indicated in the audacious title of their paper "The cure of Hodgkin's disease" [63]. Thus, the term cure began to be used with confidence in discussing radiotherapy of early stage Hodgkin's disease, although the concept did not enter textbooks of medicine for another decade, such was the pessimism about the curability of cancer [61]. Peters was generous in her tributes to Gilbert, who developed the strategy for curative radiotherapy, and Richards, who treated most of the patients with an advanced (for the day) instrument. She shared credit with him for the insightful analysis expressed in her important paper (Table 37.2).

Table 37.2 Chronology of advances in the treatment of Hodgkin lymphoma

Year	Event	Citation
1902–1903	First cases of Hodgkin's disease treated with X-rays	Pusey [56], Senn [57]
1939	Anatomic and clinical foundations, and principles of radiation therapy of Hodgkin's disease established	Gilbert [58, 59]
1940	Long-term follow-up suggested cure of some patients with radiation therapy	Craft [60]
1946	Nitrogen mustard treatment of Hodgkin's disease	Goodman et al. [64], Jacobsen et al. [65], Wilkinson and Fletcher [66]
1950	Apparent cure of Hodgkin's disease by 400 kv radiation therapy of affected and adjacent, apparently uninvolved, lymphoid areas	Peters [37]
1962	Improved technique: tumoricidal dose, megavoltage equipment, irradiation of lymphoid areas in continuity (mantle and inverted Y radiation fields)	Kaplan [67, 68]
1962	Confirmation and extension of evidence for cure of early stage Hodgkin's disease by extended field megavoltage radiotherapy	Kaplan [68]
1963	Confirmation and extension of evidence of cures of early stage Hodgkin's disease with extended field megavoltage radiotherapy	Easson and Russell [63]
1970	Cure of advanced Hodgkin's disease with mechlorethamine, Oncovin (vincristine), prednisone, procarbazine (MOPP) chemotherapy	Devita et al. [69, 70]
1975	Treatment of Hodgkin's disease with noncross-resistant drugs doxorubicin (Adriamycin), bleomycin, vinblastine, decarbazine (ABVD), and with alternating MOPP and ABVD	Bonnadonna et al. [71, 72]
1984	Autologous hematopoietic stem cell transplantation for Hodgkin's disease	Spitzer et al. [73]
1985	Allogeneic hematopoietic stem cell transplantation for refractory Hodgkin's disease	Appelbaum, Thomas et al. [74]
1997	Seven drug chemotherapy introduced (BEACOPP)	Diehl et al. [75]

By the late 1950s, orthovoltage therapy had been replaced by megavoltage (MV) therapy. These machines included a Van De Graff electrostatic generator with a peak energy of 2 million electron volts, the radioactive cobalt-60 teletherapy unit emitting gamma rays with an energy equivalent of 3 MV, the betatron providing beams of electrons or X-rays at energies of approximately 30 MV and linear electron accelerators that provide electron and X-ray beams in the 6 MV energy range. Henry Kaplan (1918–1984), a radiotherapist, working at Stanford University had begun to use a 5 MV linear accelerator in the late 1950s and 1960s. The instrument was developed by engineers at Varian Company. Kaplan was collaborating closely with the internist-oncologist Saul Rosenberg (b.1927) [76]. Rosenberg and colleagues wrote a paper, published in the journal *Medicine* in 1961, describing the finding in 1269 patients with lymphoma. This report provided important descriptive information on the disease. Kaplan and Rosenberg carefully studied and tracked the spread of disease in patients and established more convincingly the early view of Greenfield expressed in 1878 [29] that the disease principally spread contiguously in a more-or-less orderly manner [77].

With their advanced devices, Kaplan and colleagues could apply high-dose extended field radiation to treat overt and unapparent involved neighboring areas. This approach evolved from the work of Gilbert and Peters. Their results with stage I and II patients were excellent and they extended radiotherapy to patients with more extensive disease. They were able to give radiotherapy at fractionated doses in the neighborhood of 4500 rads to involved areas in the upper body and lower body with acceptable toxicity to normal tissues. The radiation-absorbed dose (rad) had replaced the Röntgen (r) for calculating radiation dose. The latter was the dose measured by the ionization produced in air, whereas the former was the dose absorbed by the tissue being treated. This was particularly important as megavoltage instruments were generating beams that had their peak dose below the skin surface. Kaplan and his team developed approaches to treat the upper and lower body in fields known as the mantle (upper and lower neck, clavicular area, axilla and mediastinum) and inverted Y fields (para-aortic area, spleen, pelvic femoral, and iliac areas). The effort was aimed at curing patients with more widespread disease or markedly extending survival by defining and delivering a dose of radiation calculated to minimize relapse [67]. In his classic paper in 1962, Kaplan described his early results and, thereafter, was the leading authority on the treatment of Hodgkin lymphoma with radiation [68]. He wrote his *magnum opus* on Hodgkin disease in 1972 and published a second edition in 1980 that encompassed every aspect of the disease and detailed progress over the 150 years since Hodgkin's classic paper [19].

Chemotherapy

Alkylating Agents

The introduction of rational chemical therapy for the treatment of Hodgkin's disease (and other lymphoid and nonlymphoid tumors) sprang from military or paramilitary research, as has often been the case in medicine. To break the stalemate that existed during World War I, the Germans used artillery shells containing dichlorethylsulfide (sulfur mustard gas) at Ypres, resulting in casualties, deaths, and terror among the allied troops, but no influence on the outcome of the battle. The postmortem studies on the mustard gas victims showed not only epithelial vesication but destruction of tissues with rapid cell turnover, such as gastrointestinal epithelium [78]. In the periods between the World Wars, James Ewing who had served in the Army during World War I encouraged the study of the effect of mustard gas on neoplastic tissue. Frank E. Adair (1888–1982) and Halsey J. Bagg (1890–1947) at the Memorial Hospital for the Treatment of Cancer and Allied Diseases in New York City followed his suggestion and studied the application of sulfur mustard gas to treat human skin tumors [79]. Adair was the chief of the breast service, a very prominent cancer surgeon, and had been president of the American Cancer Society; Bagg was a cancer research biologist at Memorial Hospital.

At the outbreak of the Second World War, with the fear that Germany would again resort to poison gas, the United States Army and the Scientific Office of the British Commonwealth formed governmental structures and committed resources to support research into the biological effects and mechanism of action of mustard gas to prepare their armed forces for a chemical weapons attack. In the United States, the studies were under the aegis of the National Research Council's wartime committees, advisory to the Surgeon General. One of the committees was the Treatment of Gas Casualties under the chairmanship of Milton C. Winternitz (1985–1959). Winternitz had been professor of pathology at Yale and then Dean of the Yale School of Medicine [80]. In 1917, World War I was raging, gas warfare had begun, and Winternitz saw the need for biological study of these new agents. His appearance before an Advisory Committee of the Secretary of War is described by a member, in these words: "… of all the great scientists who appeared before it, he was the only one who had the courage to state things as he saw them, had the remedies always at hand, and at times almost ruthlessly, but necessarily, brushed aside opposition, compromise and mediocrity." He obtained the permission of Yale University to establish a center for the study of the biological effects of war gases, and also an Army training school for laboratory medicine. Winternitz authored a book entitled *The Pathology of War Gas Poisoning*, published in 1920 by the Yale University Press, the year he ascended to the deanship, which he occupied for 15 years.

In 1942, a contract between the United States Government and Yale University was executed to study the effects of mustard gas. The vesicant properties of the gases limited their experimental usefulness and to circumvent this problem the Yale scientists, Louis Sanford Goodman (1906–2000) and Alfred Gilman (1908–1984) and their colleagues, synthesized nitrogen mustards (as compared to sulfur mustards) that could be injected intravenously in animals and humans. During these secret wartime studies, these investigators confirmed the cytocidal effects of nitrogen mustard on proliferating tissue, including marrow, lymph nodes, and gastrointestinal mucosa. The effects on lymphoid tissue led to collaborations with members of the Yale Department of Anatomy who were working with experimental animal models. They injected nitrogen mustard into mice with a transplanted lymphoma and observed a striking reduction in tumor size. Further animal studies led to the testing of two nitrogen mustard compounds on human subjects with Hodgkin's disease, other lymphomas, leukemias, and other cancers. They were given the wartime code names of HN_2 for methyl-*bis*(β-chloroethyl)amine and HN_3 for *tris*(β-chloroethyl)amine. The first patient treated with nitrogen mustard was at New Haven Hospital. By 1943 the number of humans with cancer, notably lymphoid cancers, studied was quite small, but a war-related tragedy extended the human observations of mustard effects on highly proliferative tissue.

On December 2, 1943, the Italian port of Bari on the Adriatic Sea, the main supply base for the British Eighth Army, commanded by General Bernard Law Montgomery (1887–1976) was the scene of a massive influx of American and Allied ships bringing in supplies to support the 15th United States Army Air Force, recently moved from North Africa to liberated southern Italy to give the United States a bomber-base more proximate to the heart of Germany and the Balkans, especially the Romanian oil fields in Ploiesti. The 15th was commanded by major General James Harold (Jimmy) Doolittle (1896–1993). Doolittle was a heroic figure and an aviation pioneer. He led the raid of 16 B-25 medium bombers on five cities in Japan including Tokyo, 4 months after Pearl Harbor (April 18, 1942). Departing from the carrier United States Ship Hornet, they did not have sufficient fuel to return. They parachuted over Japanese occupied China and were smuggled through Japanese lines to friendly Chinese forces. The bombing of Japan was an enormous boost to the beleaguered allies at that point in the war.

One of the ships in the port of Bari was a liberty ship, the John Harvey, named for a member of the Continental Congress in 1777 and a signer of the Articles of Confederation. The American ship was loaded with two thousand, one hundred pound, bombs containing mustard. The allies were concerned that the Germans in desperation would use mustard gas against their forces and they planned to be prepared to retaliate. The night of December 2nd saw an unexpected air raid by over 100 German Junkers Ju-88 bombers. The bomber attack was planned by Field Marshal Freiherr von Richthofen (1895–1945), a distant cousin of Manfred von Richthofen (1892–1918), the World War I ace, referred to as The Red Baron, with the approval of Field Marshall Albert Kesserling (1885–1960), commander of all German troops in Italy. During the day of December 2nd, a German Messerschmitt-210 reconnaissance plane photographed Bari harbor choked with ships at anchor virtually touching each other and filling the port. Upon seeing these photographs, the Germans launched the raid that night catching the Americans and British off guard. They did not believe the retreating Germans and the decimated Luftwaffe in Italy could launch such an attack. British Air Vice Marshal Arthur Coningham (1895–1948), at a press conference on December 2nd, stated defiantly that the Luftwaffe in Italy was defeated and could never attack Bari. During the bombing raid the John Harvey caught fire as a result of hits by German bombs on neighboring ships. It exploded and released mustard gas leading to extensive deaths and casualties. Examination of some of the over 600 affected merchantmen and seamen confirmed the damage to the marrow, lymphatic tissues, and gastrointestinal mucosa and, of course, skin and lungs. This horrendous tragedy, many ships sunk or severely damaged, severe disruption of the flow of supplies (the civilian casualties were never accurately counted) was referred to as "the second Pearl Harbor." The only positive result of this horrendous tragedy was that the autopsy and medical findings, which supported the small number of human studies of mustards at that time, were thought to further accelerate the use of mustard compounds in cancer treatment and notably in Hodgkin's disease patients [81]. von Richthofen died of a tumor in Allied captivity in 1945 and Kesserling was convicted and sentenced to life in prison for war crimes committed in Italy.

In April 1946, Gilman and Frederick S. Philips published in the journal *Science* [82] a detailed report of the chemistry of the mustard compounds and their tissue effects in a variety of species. They commented on their nucleotoxic effects and indicated that the tissue effects were unlike any other chemical agent but closely simulated the effects of X-radiation (radiomimetic). They mentioned the results of the first six patients treated and indicated that there were results from treating approximately 150 patients, but the data were still under wartime secrecy and although the names of the investigators were noted in the bibliography, no source was given. It was noted that the most dramatic responses were seen in Hodgkin's disease and not in lymphosarcoma or acute or chronic leukemia.

Also, in 1946, Cornelius P. Rhoades (1898–1959), Chairman of a Committee of the National Research Council, reported a summary of the results of the clinical trials of

nitrogen mustards in patients with Hodgkin's disease and other malignancies in an Official Statement to the Medical Profession. Rhoades was the Director of the Sloan-Kettering Institute for Cancer Research and the Director of Memorial Hospital. Subsequently, lifting the cloak of wartime secrecy, the three detailed reports of the effect of nitrogen mustard in human cancer were published in the same year [64–66].

Goodman and Gilman's paper is given priority based on their synthesis of the mustard compounds, extensive preclinical studies, and careful clinical evaluation in concert with Maxwell Myer Wintrobe (1901–1986) and William Dameshek (1900–1969), two of the leading hematologists of their day [64]. In their classic paper, Goodman and colleagues reported the treatment of 67 patients with Hodgkin's disease, lymphosarcoma, and acute and chronic lymphocytic and myelogenous leukemia. Most were previously treated with X-irradiation and were in the late stages of their disease. Several dramatic responses occurred, especially in cases of Hodgkin's disease [64]. This finding was also reported by Leon Jacobsen (1911–1992) and colleagues at the University of Chicago [65] and John Frederick Wilkinson (1897–1998) and Frank Fletcher (d.1995) at the Manchester Royal Infirmary in their simultaneous studies [66]. The studies of Goodman and coworkers were conducted at hospitals in New Haven, Salt Lake City, Boston, and Portland presaging multicenter collaboration in order to accumulate enough cases in a reasonable period of time to draw conclusions of efficacy and toxicity of the drug(s) under examination. Nitrogen mustard, thereafter, became a mainstay of treatment for Hodgkin's disease, and 25 years later was incorporated into a curative multidrug regimen (see discussion later in this chapter).

The nitrogen mustards were difficult to use; therapists had to protect themselves from skin contact or inhalation and the patient could suffer burns from extravasation or vein damage. For these and other reasons (e.g., high toxicity on marrow and gastrointestinal tract, inability to penetrate the blood–brain barrier or to be used intrathecally), other molecules with similar tissue effects were developed. Nitrogen mustard was a relatively simple molecule and it was easy to modify it chemically. Hundreds of derivatives were tested. Busulfan and chlorambucil were synthesized and tested at the Chester Beatty Institute in London and their use described in 1953. Cyclophosphamide was synthesized in Germany and introduced to the clinic in about 1960 [83]. Busulfan, cyclophosphamide, and chlorambucil are pro-drugs and can be administered orally, requiring conversion in the body to their active forms. The specific tissue effects of nitrogen mustard and related compounds, including DNA cross-linking and alkylation of various molecules, notably DNA, took several decades to unravel [66].

Procarbazine, a methylhydrazine derivative, has several mechanisms of action but can also directly damage DNA through an alkylation reaction. It was developed in the early 1960s and was soon recognized as an active agent in the treatment of lymphoid malignancies [84]. During the last few decades, comprehensive studies have clarified cellular pathways involved in the modes of action of procarbazine and its drug resistance mechanisms. It was approved by the Food and Drug Administration for general use in 1969 and became one of the four drugs that would eventually be used to treat and cure advanced Hodgkin's disease (see section on Combined Drug Therapy).

Glucocorticoids

In the 1920s interest in the secretory products of the adrenal cortex led to extraction of an active principal which was named "cortin." Taddeus Reichstein (1897–1996), a Polish-born, Swiss chemist working at the University of Basel, and Edwin Calvin Kendall (1886–1972), an American biochemist, working at the Mayo Clinic in Rochester, Minnesota, were interested in characterizing this material. Reichstein used extraordinary numbers (thousands) of bovine adrenal glands from which to extract and prepare very small amounts of semi-purified material. He identified six principal constituents, which were further characterized by Kendall who named them compounds A through F. Compound E, later identified and named cortisone, and synthesized by Kendall in 1948, produced the most dramatic shrinkage of thymus and lymph nodes in experimental animals and could retard or even prevent the development of lymphoid tumors transferred to host animals. Adrenocorticotropic hormone produced similar results but could not generate the levels of cortisone in vivo that could be administered therapeutically and, as a protein, required injection. The limited supply of cortisone made human studies difficult. Kendall and Philip Showalter Hench (1896–1965) invited the collaboration of the Merck Company, and soon after World War II, synthetic material became available for human studies. This cooperation marked one of the earliest collaborations between an academic medical institution and a pharmaceutical company. As an aside, the dramatic anti-inflammatory effect on persons with severe rheumatoid arthritis and their near miraculous relief of pain and restoration of joint motion described by Kendall, Hench, Charles H. Slocumb (1905–1996), and Howard F. Polley (1913–2001) in 1949 led to Kendall, Hench, and Reichstein sharing the Nobel Prize in Physiology or Medicine in 1950 for the discovery and synthesis of cortisone and its use in ameliorating human disease. One year earlier in 1949, Olof H. Pearson (d. 1990) and Leonard Paul Eliel (1914–2004), working in the Division of Clinical Investigation, Sloan-Kettering Institute for Cancer Research and the Department of Medicine, Memorial Hospital, New York, published the usefulness of cortisone in the treatment of lymphocytic malignancies [85]. A cortisone derivative

would provide the third (mechlorethamine, procarbazine, and cortisone) of the four agents (plus vincristine) that would eventually cure advanced Hodgkin's disease. Subsequently more potent congeners of cortisone such as prednisone and dexamethasone were synthesized, and became the glucocorticoids of choice in the treatment of lymphoid malignancies. The first commercially feasible synthesis of prednisone was carried out in 1955 in the laboratories of Schering Corporation by Arthur Nobile (1920–2004) and coworkers. They discovered that cortisone could be oxidized microbiologically to prednisone by the bacterium *Corynebacterium simplex*. The same process was used to prepare prednisolone from hydrocortisone. Prednisone and prednisolone were introduced by Schering in the mid-1960s.

Vinca Alkaloids

Vinblastine, extracted from the Madagascan periwinkle *Catharanthus roseus*, was first discovered as an unexpected myelosuppressive agent in 1958 by Robert Laing Noble (1910–1990) and colleagues working in Canada during their search for an antidiabetic agent [86]. Independently, researchers at Eli Lilly Company found that extracts of *C. roseus* possessed activity against P-1534 leukemia in mice and isolated vinblastine as its active entity in 1959. The structure of a related compound, vincristine methiodide, was then determined by X-ray crystallography in 1965. Structural studies on these alkaloids revealed that both vinblastine and vincristine were bisindole alkaloids. These alkaloids inhibit assembly of tubulin units into microtubules, resulting in the arrest of cell division at metaphase. Although these compounds are very similar in structure and exhibit the same mode of action, they have different effects. Vincristine is most useful in acute lymphoblastic leukemia and Hodgkin's disease and other lymphomas and would be the fourth of four drugs used in combination to cure Hodgkin's disease (see next section). In 1993, the National Cancer Institute of Canada initiated the Robert L. Noble Prize (not to be confused with the Nobel Prize), which is awarded each year to the investigator whose contributions have led to a significant advance in cancer research. Noble was awarded the Gairdner Foundation International Award in 1984 and was inducted into the Canadian Medical Hall of Fame, posthumously, for his scientific contributions.

Combined Drug Therapy

Combining drugs for the treatment of disease was anathema among prominent internists and hematologists in the 1950s and early 1960s. It was considered reasonable to combine drugs to treat *Streptococcus viridans* subacute bacterial endocarditis (penicillin and streptomycin) or *Mycobacterium tuberculosis* (isoniazide, streptomycin, and the para-amino salt of salicylic acid) but not for other diseases. Indeed, a classic study of penicillin and tetracycline for pneumococcal meningitis showed a worse outcome with both drugs compared to penicillin alone. This reinforced the proscription to combining drugs, championed by the purists. At a Blood Cell Club meeting at the Hadden Hall Hotel in Atlantic City in the mid-1960s, the leading hematologists in the United States, aggressively castigated the group working at the National Cancer Institute, studying four drug combinations for acute lymphoblastic leukemia in children, a vituperative dispute that raged for a time: the oncology pioneers against the establishment. Over time, the utility of drug combinations in the treatment of malignant diseases, notably acute childhood lymphocytic leukemia, Hodgkin's disease, and lymphoma became irrefutable.

The initial studies of multidrug combinations in the treatment of advanced Hodgkin's disease began at the National Cancer Institute in Bethesda, Maryland. It had been preceded by studies of various single drugs, nitrogen mustard, cyclophosphamide, vinblastine, and vincristine. Several principals were established. One was that complete remission was an important goal as these patients fared better than those who did not enter a complete remission. This finding was already apparent to other investigators in the National Cancer Institute studying childhood acute leukemia. The first pilot protocol of combination therapy included vincristine, methotrexate, cyclophosphamide, and prednisone (the MOMP regimen) given for 2.5 months [87]. Vincristine was selected over vinblastine because it had less marrow toxicity and at that time support for patients with hematopoietic suppression was primitive. There were no indwelling catheters, no effective means of platelet transfusion, and a more limited array of antibiotics. Procarbazine had been shown to be an active agent in Hodgkin's disease by then and it replaced methotrexate in the next step. Each drug was used at its optimal dose and a cycle of administration was selected as every 28 days, the shortest interval that would allow marrow recovery between cycles. In 1967, David P. Rall (1926–1999), then chief of the Laboratory of Chemical Pharmacology at the National Cancer Institute and a mentor of Vincent DeVita, said to a colleague, "I just got back from an Institute intramural meeting at which the preliminary results of Hodgkin's disease treatments were being discussed, and it looks as if DeVita is going to cure Hodgkin's disease with MOPP before you guys cure childhood ALL." Vincent T. DeVita, Jr. (b. 1935) and colleagues, working at the National Cancer Institute, introduced the first highly effective combination chemotherapy for the treatment of advanced stages of Hodgkin's disease. The trial of this four-drug combination, initiated in 1964, **m**echlorethamine (nitrogen mustard), vincristine (**O**ncovin), **p**rocarbazine, and **p**rednisone, carrying

the acronym MOPP, was developed based on experimental studies indicating the desirability of combining agents with different mechanisms of action and with nonoverlapping toxicities. This drug combination, the results of which were reported in an abstract in 1967 and in a paper in 1970, extended curative treatment to the advanced stages of Hodgkin's disease [69]. However, with longer periods of observation late toxicities, especially secondary cancers and sterility, resulted in the search for drug combinations with a better safety profile [88, 89]. A substitute four-drug approach was introduced by Gianni Bonnadonna (1934–2015) and coworkers, called by its abbreviation ABVD, describing the four drugs, doxorubicin (*A*driamycin), *b*leomycin, *v*inblastine, *d*acarbazine, which produced a better response rate and a lower frequency of secondary leukemias and other serious complications than MOPP [71, 72]. Procarbazine, although earlier withdrawn from combination regimens used to treat Hodgkin's lymphomas, has reappeared in dose-intensified regimens and has yielded impressive results. A seven-drug regimen bleomycin, etoposide, doxorubicin, cyclophosphamide, vincristine, procarbazine, and prednisone referred to by the acronym BEACOPP or in varying doses, escalated BEACOPP, is being compared to ABVD for efficacy and safety [75].

Hematopoietic Stem Cell Transplantation

Although most patients with Hodgkin lymphoma can be cured with chemotherapy or radiation therapy, a modest proportion are refractory to therapy or relapse after entering a remission. Edward Donnall (Don) Thomas (1920–2012) and colleagues at the Fred Hutchinson Cancer Research Center presented early results of the use of stem cell transplantation for this disease [74]. Thomas shared the Nobel Prize in Physiology or Medicine in 1990 for his pioneering and singular research leading to the development of syngeneic and allogeneic hematopoietic stem cell transplantation for the curative therapy of aplastic anemia and hematological and lymphoid malignancies. His co-awardee was Joseph Edward Murray (1919–2012), a pioneer in developing the technique of syngeneic, allogeneic, and cadaveric kidney transplantation. They had been residents together at the Peter Bent Brigham Hospital in Boston. At about the same time that Thomas was exploring allogeneic transplantation for refractory or relapsed Hodgkin's disease, Gary Spitzer (b. 1944) and others at the University of Texas M.D. Anderson Hospital and Tumor Institute showed the potential usefulness of high-dose chemotherapy with autologous marrow stem cell infusions to restore hematopoiesis more rapidly [73]. Spitzer's office at M.D. Anderson Hospital was on a patient floor and he used the patients' coffee dispenser. One day at the coffee machine he was chatting with the mother of a patient in the hospital with her sixth relapse of Hodgkin's disease and with pulmonary involvement. The mother asked Spitzer what he did and after learning of his interest in transplantation suggested he transplant her daughter. The daughter's physician was supportive and Spitzer did his first autologous transplantation in Hodgkin disease after conditioning the patient with cyclophosphamide, 1,3-*bis*(2-chloroethyl)-1-nitrosourea (BCNU), and etoposide (VP-16). The patient's pulmonary nodules gradually disappeared and she had no return of disease, thereafter. This experience propelled Spitzer's autologous transplantation program for Hodgkin's disease. High-dose chemotherapy followed by autologous blood or marrow stem cell infusion to rescue the patient from potentially lethal therapeutically induced prolonged marrow aplasia is the current standard of care. Currently, the use of allogeneic stem cell transplantation with the latter's theoretical benefit of graft versus lymphoma effect and a potentially more robust stem cell product is a matter of debate in these heavily pretreated patients.

Historical Concepts of Lymphoma

Early Descriptions

The first descriptions of primary tumors of the lymph nodes, as specifically distinguished from Hodgkin lymphoma, can be attributed to Theodor Billroth (né Christian Albert Theodor Billroth) (1829–1894). Billroth was trained in internal medicine, experimental and anatomic pathology, and abdominal surgery. He published important papers in pathology and surgery. His was the first accurate description of the red pulp of the spleen, describing the sinus structure and cords, later called the sinuses and cords of Billroth. He competed with Virchow for the chair in pathology at the University of Berlin, but his outspoken opposition to unproven hypotheses promulgated in the authoritarian professorial system in Germany contributed to his losing that opportunity.

Billroth was an accomplished, indeed gifted, pianist, later took up the viola so that he could participate in string quartets, and was a student of music. His maternal grandparents were opera singers. He published a book on music and was a close confidant of Johannes Brahms (1833–1897). Brahms dedicated two of his three string quartets (the C minor and A minor quartets, Op. 51) to Billroth and gave him the original manuscripts of the music. Billroth had continued his work on the piano, intensively, while in medical school at the University of Göttingen and on one occasion provided piano accompaniment to Johanna Maria (Jenny) Lind (1820–1887), the Swedish soprano (dubbed the "Swedish Nightingale"), during an appearance in Germany [90, 91]. In Vienna, his relationship with Brahms grew, as the latter had

moved there also. Throughout the 1870s, the chamber music composed by Brahms was first played in Billroth's home before a select audience.

Billroth became one of the most distinguished surgeons of his time, practicing, teaching, and writing in Zurich, and then Vienna, and was one of the great pioneers of surgery, especially gastric and other abdominal surgery. As professor of surgery in Vienna he collaborated with Alex von Winiwarter (1848–1917), professor of surgery in Lüttich, and wrote a classic text of the day, *Die allgemeine chirurgische Pathologie und Therapie* (General Surgical Pathology and Therapy) in 1863 [92]. In Chap. 48 of this 1000 page text, he discusses the "Sarcome," which included a discussion of "Benigne and maligne lymphoma" and "Sarkome der lymphdrüsen." The term "sarcoma" has been attributed to Galen, who coined it to describe a soft tissue tumor. In a detailed paper published earlier in 1861 covering several topics related to lymph node pathology, Billroth devotes a section to "Sarcom der lymphdrüsen" as distinguished from "Die carcinomatösen lymphdrüsen" and other afflictions involving lymph nodes [93]. A later paper by Hans Kundrat (1845–1893) entitled "Uber Lympho-sarkomatosis" in 1893 [94] has been credited, apparently erroneously, for the first use of the term, lymphosarcoma. Kundrat was a younger colleague of Billroth and, it can be inferred, that he was introduced to the concept by Billroth. Indeed, Billroth reviewed Kundrat's paper for the *Wiener klinische Wochenschrift*. Nevertheless, Kundrat presented several cases, discussed the variations in the presentation of lymphosarcoma, and tried to distinguish it from the nonspecific term pseudoleukämie and some cases of Hodgkin's disease, which was an important addition to the relatively skimpy understanding of lymphoma at that time. He described the spread of the disease to other lymph node sites, involvement of liver and spleen, and local invasion of neighboring tissues, distinguishing "lymphophosarkom" from "lymphosarkomatosis." Cases in which the neoplastic cells remained confined to the lymphatic system were described also by others soon after, but as others before him, Kundrat did not have histopathological methods to distinguish Hodgkin's disease from other lymphomas with any certainty [94]. For a time, lymphosarcoma was called "Kundrat's disease" in Europe.

Virchow discussed lymphoma and lymphosarcoma quite extensively at about the time that Billroth did [95]. In his section on lymphosarkome, he discussed thoracic and abdominal spread and compression of mediastinal structures. However, in 1895, Virchow discussed cancer of various types in his lectures and referred to the splenic and lymphatic forms of leukemia [10]. In both the lymphatic and splenic form of leukemia, he emphasized the role of a large spleen. He stated, "Now, upon investigating whence this curious change in the blood takes its origin, we find in the great majority of cases that it is a certain, definite organ which presents itself over and over again with convincing constancy as the one essentially diseased, an organ which frequently, even at the onset of the malady, forms the chief object of the complaints and distress of the patients, namely, the *spleen*. In addition, a number of lymphatic glands are very frequently diseased, but the affection of the spleen stands in the foreground." From the descriptions, it is probable that he is describing chronic lymphocytic leukemia and chronic myelogenous leukemia, respectively, in his lymphatic and splenic forms. Nowhere is a primary tumor of the lymph nodes discussed although acute lymphadenitis and tuberculosis involving the lymph nodes were described. To the extent that chronic lymphocytic leukemia is analogous to small-cell lymphocytic lymphoma, Virchow's observations were seminal but the state of knowledge of lymphohematopoiesis, the inability to stain blood cells, and the late stage at which patients were assessed make interpretations of the descriptions of the day largely inferential. Because of Virchow's dominance in pathological thought at that time, the concept that tumors of lymph nodes arose from connective tissue cells, which he espoused, was slow in being replaced. Billroth had reluctantly accepted that his own concept of connective tissue as the source of lymphatic cells was in error. Virchow was less tractable.

By the early twentieth century burgeoning interest in lymphoma developed. Textbooks of pathology had entries in their table of contents recognizing lymphomas as a discrete type of cancer. In the text book published in 1914 by Frank Barr Mallory (1862–1941), pathologist at the Boston City Hospital and associate professor at Harvard Medical School, a 159-page section on "Tumors" contained seven pages describing "lymphoblastomas" with the parenthetical designations of lymphocytoma, lymphoma, lymphosarcoma, pseudoleukemia, lymphatic leukemia, and Hodgkin's disease [96]. He provided a description of large lymphoblasts found in the germinal centers of lymphatic structures, small lymphocytes, and the derivation of plasma cells from lymphocytes. He coupled Hodgkin disease with the scirrhous form of lymphoblastoma and, remarkably, felt that "The term Hodgkin's disease was inexact and should never be used. It always covers ignorance of the true nature of the lesion." This conclusion was reached despite Dorothy Reed's definitive work showing Hodgkin's disease to be a distinct entity a decade before and may reflect the absence of a unified medical reference source to search for recent papers. On page 329, Fig. 246 of his text [96], Mallory shows a drawing in color of the histological pattern of one form of lymphoblastoma that appears to be a case of Hodgkin lymphoma, including eosinophils and two giant tumor cells resembling Reed–Sternberg cells. On page 327, Fig. 243, he shows a histopathological replica of a lymph node containing a "lymphoblastoma growing rapidly," containing lymphoblasts with prominent nucleoli and frequent mitotic figures.

By the early twentieth century, cellular pathology had advanced and histopathological classification of a wide variety of human benign and malignant neoplasms had developed, the evolution of cancer as a specialized area of scientific and medical study was initiated, and the treatment of cancer by surgery, radiation therapy, and chemicals (principally arsenicals) was in its rudimentary stages.

In his textbook, Ewing (discussed earlier in this chapter) included a chapter entitled "Lymphoma and Lymphosarcoma," a topic to which he devoted 43 pages encompassing descriptions of various types of lymphocytic tumors [51]. He classified one such tumor as an endothelioma of lymph nodes. In an additional chapter, he described "The Thymus and its Tumors." Ewing was the leading American experimental oncologist of his day. In 1907, he helped found the American Association for Cancer Research, and in 1913, the American Society for the Control of Cancer, now the American Cancer Society.

In 1931 Ewing was elected to the presidency of the Medical Board of the General Memorial Hospital for the Treatment of Cancer and Allied Diseases, and became its director. (The designation "General" was dropped in 1916.) It was the earliest privately owned hospital devoted to cancer diagnosis and treatment and one of the earliest hospitals of any kind in the United States dedicated to the sole purpose of caring for cancer patients. It began in 1884 as The New York Cancer Hospital on the upper West Side of Manhattan, changed its name to the Memorial Hospital for the Treatment of Cancer and Allied Diseases (usually referred to as the Memorial Hospital), and moved to the East Side in 1939 [97]. In 1945 (Ewing had died 2 years earlier), Alfred P. Sloan, Jr. (1875–1966), Chairman of the General Motors Corporation, gave four million dollars to establish the Sloan-Kettering Institute for Cancer Research. Although geographically proximate to the Memorial Hospital, they did not become a unified corporate entity until 1960 when the Memorial Sloan-Kettering Cancer Center was established. Charles Kettering (1876–1958) was one of the great American innovators and inventors of the twentieth century. His many inventions resulted in the advancement of the automobile industry along with other contributions, such as refrigeration. His development of the electric automobile starter, first installed in Cadillacs in 1912, replacing the crank starter, revolutionized the automobile industry and propelled General Motors to the forefront of vehicle manufacturers. In 1945, Kettering helped Sloan found the Sloan-Kettering Institute for Cancer Research, later to become the Memorial Sloan-Kettering Cancer Center. His support was based on the premise that American industrial research techniques could be applied to cancer research.

Although a few centers devoted to cancer research and care developed in industrialized countries, including the United States, these represented a rudimentary approach to cancer treatment until the early 1970s. Most cancer patients were cared for by general surgeons or internal medicine specialists. Medicine was traditionally organized around organ systems. For example, nephrologists managed renal cancer and gastroenterologists managed gastrointestinal cancers. General surgeons excised cancers and administered the limited chemotherapy available for nonhematological cancers (e.g., 5-fluorouracil) and hematologists cared for patients with leukemia and lymphoma. Specialized therapists treated cancer by irradiation. The impetus to make oncology a broadly accepted discipline, a discipline that involves all organ systems, was provided by Richard Milhous Nixon (1913–1994), the 37th President of the United States. In his State of the Union address in 1970, he committed the government to decreasing and eventually eliminating the threat of cancer. As part of this national effort, in October 1971, the Army's Fort Detrick biological warfare facility in Maryland was converted to a cancer research center, eventually becoming the Frederick Cancer Research and Development Center, an internationally recognized center for cancer and, later, AIDS research. On December 23, 1971, President Nixon signed into law the National Cancer Act, declaring, "I hope in the years ahead we will look back on this action today as the most significant action taken during my Administration."

The National Cancer Act (Public Law 92–218), dubbed "The War on Cancer," gave the National Cancer Institute autonomy at the National Institutes of Health with special budgetary authority to be determined by Congress. The Cancer Chemotherapy National Service Center increased its efforts to acquire new compounds for testing with the awarding of an acquisition and inventory contract responsible for the collection and documentation of test agents. In 1973, the Surveillance Epidemiology and End Results Program of the National Cancer Institute was initiated as an authoritative source of information on cancer incidence and survival in the United States. The Program, known by the acronym SEER, currently collects and publishes cancer incidence and survival data from population-based cancer registries. Since cancer is not a reportable disease in the United States, this initiative was vital to measure the incidence, prevalence, and mortality of cancer and to track changes in incidence and mortality and, thus, measure progress. This agency confirmed the dramatic increase in lymphoma incidence (but not Hodgkin lymphoma incidence) between 1973 and 1995, which was seen in most First World (industrialized) countries, probably starting in the late 1940s, and still unexplained. In 1972, it was reorganized to consist of the office of the director and four divisions: cancer biology and diagnosis, cancer cause and prevention, cancer treatment, and cancer grants (later named division of extramural activities).

The increased focus on and money for cancer research resulted in the Cancer Centers Program of the National Cancer Institute. In 1973, the director of the National Cancer

Institute, Frank J. Raucsher, Jr. named eight institutions the first comprehensive cancer centers. Additional centers were funded thereafter. There was expansion of Institute-funded, cooperative, and multicenter cancer clinical trial groups. One of the most influential leaders at the National Cancer Institute from the mid-1950s through the mid-1970s was Charles Gordon Zubrod (1914–1999), who modeled the new attack on the leukemias after the highly successful malarial treatment and prophylaxis program he was involved with during the Second World War. He likened the attack on malaria, vital to the allies' success in the Asian theater of war, to the Manhattan Project in its organization. The medical scientific community was not enamored of targeted research, preferring investigator-initiated approaches, but Zubrod thought both approaches were needed so he set up the Acute Leukemia Task Force at the Cancer Institute [98]. He started the concept of regional multicenter groups, such as the Eastern Cooperative Oncology Group and the Southwestern Oncology Group. The infusion of money and concepts led to attractive opportunities for scientists and physicians to focus on the understanding and management of patients with cancer as well as its causation and pathogenesis. These initiatives fostered the development of the specialties within oncology (e.g., medical, pediatric, gynecologic, surgical, and radiation oncology), the development of departments of neoplastic diseases, the development of cancer centers in university medical centers, the expansion of basic research relevant to cancer genetics and cytogenetics, and the study of the pathobiology of cancer cell growth and survival.

Appreciating the Histopathological Heterogeneity and Attempts at Classification

The terms reticulosarcoma and reticuloendotheliosarcoma were first used by Charles Oberling (1895–1960) [99], likening it to the reticuloendothelioma of Ewing. Oberling believed that these tumors arose in marrow and that they were analogous to or derived from the same cells as tumors arising in lymph nodes. Some of these cases were likely tumors of bone. Oberling was one of the most highly regarded pathologists of his period. He was chair of microbiology and hygiene in Strasbourg where he worked on viral origins of cancer, having been influenced by Amédée Borrel (1867–1936), a French biologist. Borrel is considered the father of the virus theory of cancer and was also a distinguished microbiologist for whom the genus of bacteria *Borrelia* was named. Frédéric Charles Roulet (1902–1985), working in Switzerland [100], reinforced the use of the term "retothelsarcom" for a certain histological type of large-cell lymphoma, but his descriptions were vague and not sufficiently reproducible to separate it from other variants of lymphoma. Nevertheless, his concept of small- and large-cell lymphoma and reticulosarcoma had the seeds of a categorization. These terms did not enlighten, since no one actually knew what a reticulum cell was. The term was, therefore, transient because it did not describe accurately the cells involved; reticulum (reticular) cells or endothelial cells, after all, are not part of the cell clone in lymphoma.

It was Nathan Edwin Brill (1860–1925) who made the first and a lasting contribution to our understanding of the varied histopathological manifestations of lymphoma by identifying and specifying the distinction of lymphomas with a follicular pattern in the lymph nodes in his publication in 1925 [101]. Although Brill described only two cases, his histopathological and clinical descriptions and the effects of radiation therapy on his two patients were important findings at the time. He distinguished the lymph node pathological changes from benign lymphoid hyperplasia. Brill joined the staff of Mount Sinai Hospital in New York City after graduating from New York University College of Medicine and interning at Bellevue Hospital. He later became a professor of medicine at Columbia University College of Physicians and Surgeons. Thirteen years later in 1938, Douglas Symmers (1879–1952), a pathologist at Bellevue Hospital in New York City, described similar pathological findings in seven cases, and indicated that it was not an uncommon disease, thereby bringing increased attention to this form of lymphoma [102]. He concluded that giant follicular lymphadenopathy with or without splenomegaly was a distinct clinical and pathological entity. He also indicated that it could transform to a more malignant lymphoma phenotype. He recommended that the term "reticulum cell lymphosarcoma" be abandoned. He felt no such tumor existed. Giant follicular lymphoma was for a time referred to as Brill–Symmers disease. In effect, this was the beginning of lymphoma classification with the appreciation that follicular lymphomas represented a distinct subset of lymphoma.

In 1934, George Russell Callender (1884–1973) tried to develop a classification of the lymphomas from the several hundred cases collected by the Lymphocyte Tumor Registry of the American Association of Pathologists and Bacteriologists, established in 1925 and later moved to the Army Medical Museum, under the auspices of the National Research Council, and renamed the Lymphatic Tumor Division of the American Registry of Pathology. Callender tried to fuse three features: cytology, gross anatomy, and clinical distribution. The material was inadequate to do so and he continued to use the term "reticulum cell sarcoma" for some cases, lymphosarcoma, and giant follicular lymphadenopathy (of Brill and Symmers) for others [96]. Callender considered the reticulum cell a type of histiocyte. Callender had a military career. He served as pathologist at the Army Medical Center and wrote the monograph *Malaria in Panama*. He was Commandant of the Medical Professional Services Schools from 1940 to 1946 and retired as Brigadier General, having received the Distinguished Service Medal (Table 37.3).

Table 37.3 Evolution of the classification of lymphoma

Year	Classification	Comments and references
1861	Discussion of "Sarcom der lymphdrüsen" versus "Die carcinomatösen lymphdrüsen"	Billroth [93]
1863	*Die allgemeine chirurgische Pathologie und Therapie* (General Surgical Pathology and Therapy). Chapter 48, contains a discussion of "Benigne and maligne lymphoma" and "Sarkome der lymphdrüsen"	Billroth and Winiwarter's textbook [94]
1893	"Uber Lympho-sarkomatosis" Kundrat's paper discussing clinical presentations of "lymphosarkom" and "lymphosarkomatosis	[95]
1894	Lymphome and lymphosarcoma. Virchow's discussion of lymphoma	[96]
1914	*The Principles of Pathologic Histology*. Textbook by Frank B. Mallory. Describes "lymphoblastomas" with the parenthetical synonyms of lymphocytoma, lymphoma, lymphosarcoma, pseudoleukemia, lymphatic leukemia, and Hodgkin's disease	[97]
1925	Description of giant lymph follicle hyperplasia (follicular lymphoma) by Brill	[100]
1934	Giant follicular lymphoma, lymphosarcoma, reticulum cell sarcoma distinguished by Callender	[103]
1938	Expanded description of giant follicular lymphadenopathy by Symmers	[102]
1942	Gall and Mallory classified lymphomas into seven categories: two types of reticulum cell sarcoma (stem-cell lymphoma and clasmatocytic lymphoma), lymphoblastic lymphoma, lymphocytic lymphoma, follicular lymphoma, Hodgkin's lymphoma, and Hodgkin's sarcoma	[104]
1956	Rappaport highlights prognostic distinction between nodular and diffuse lymphomas	[105]
1966	Rappaport's morphological classification. Lymphocytic and histiocytic; large and small cell (see text for details)	[106]
1974	Kiel classification (morphology and immune markers)	[107]
1974	Lukes and Collins classification (morphology and immune markers)	[108]
1982	Working formulation (morphology and response to therapy)	[109]
1988	Updated Kiel classification	[110]
1994	Revised European-American Lymphoma (REAL) classification (morphology, biology, clinical features)	[111]
2001 (revised 2008, 2016)	World Health Organization classification	[112]

Further details of these progressive steps are discussed in the text of the chapter

In 1942, a proposal for classification of lymphomas was made by Edward A. Gall (1906–1979) and Tracy Burr Mallory (1896–1951) based on a clinicopathological survey of 618 cases [104]. Mallory, the son of Frank B. Mallory (1862–1941), a pathologist at the Boston City Hospital and Harvard Medical School, had succeeded James Homer Wright (1869–1928) as chief of pathology of the Massachusetts General Hospital in 1926 and Gall was, at that time, Chairman of Pathology at the University of Cincinnati School of Medicine. Working together, they felt it was relatively easy to divide all lymphomas into seven categories: two types of reticulum cell sarcoma (stem cell lymphoma and clasmatocytic lymphoma), lymphoblastic lymphoma, lymphocytic lymphoma, follicular lymphoma, Hodgkin's lymphoma, and Hodgkin's sarcoma. Clasmatocyte was a term used as a synonym for histiocyte. Here, as in Callender's efforts, we see the inability to distinguish certain types of malignant lymphocytes from histiocytes by microscopy alone.

A decade later, in the 1950s, Henry Rappaport (1913–2003) in a study of 253 carefully selected pathological specimens from the Lymphatic Tumor Registry of the Armed Forces Institute of Pathology archives described the features of several variations of follicular lymphoma, providing a detailed list of criteria to distinguish follicular lymphoma from follicular hyperplasia in the overwhelming proportion of cases [105]. By this time it had been appreciated that on average follicular patterns in histopathological lymph node specimens predicted for a more indolent course of the disease. Henry Rappaport was affiliated with the Armed Forces Institute of Pathology and Veterans Administration Central Laboratory for Anatomical Pathology. He later moved to the University of Chicago, Department of Pathology. Rappaport also developed a classification of non-Hodgkin's lymphomas based largely on histological criteria [106]. It was a milestone in the description of lymphoma phenotypic heterogeneity and became the most widely used classification of its day. The reticulum cell was thought to be a histiocyte by Callender, Gall, and Rappaport. Rappaport's classification also had a category of histiocytic lymphoma (né reticulum cell sarcoma), which was pathobiologically unsupportable because lymphomas are by definition diseases of a clone of lymphocytes and histiocytes are myeloid cells that develop from an unrelated lineage (histiocytoses). This paradox seemed not to bother these otherwise distinguished experts. Rappaport, also, questioned whether the follicles in follicular lymphoma were part of the neoplastic process and suggested that one use the terms "nodular" and "diffuse," the former a substitute for follicular. His categorization of lymphoma included two histopathological patterns in lymph nodes, nodular or diffuse proliferations, and four cytological patterns: well-differentiated (small cell),

poorly differentiated (large cell), and histiocytic, or a mixture of lymphocytic and histiocytic. It was attractive in its relative simplicity and had a strong relationship to histopathology and to the rate of clinical progression. Small cell nodular had the slowest rate of progression, and large cell diffuse the fastest. But, Rapport's classification was limited profoundly by the insensitivity of microscopic pathology not supplemented by other more specific and sensitive techniques. In addition, by the 1970s, at least six classifications of lymphoma were used in different parts of the world. This limited the power of international collaboration in interpreting and comparing treatment trials.

Development of Immunological Markers

For the first 55 years of the twentieth century progress had been made in the microscopic anatomy of lymphatic structures, the differences in the expression of immediate and delayed immune reactions, and the understanding of antibody production, but the functional organization of the immune system and the genetic and functional heterogeneity of lymphocytes had not been uncovered.

The bursa of Fabricius was named after the Italian anatomist Hieronymus Fabricius who described it as a result of his elaborate animal dissections in 1621. He also was an early expert in fetal anatomy and is considered by some as the first embryologist. William Harvey studied with Fabricius who was professor of anatomy and surgery in Padua. In 1955, an unexpected event dramatically accelerated the understanding of the organization of the immune system. Hens that had the bursa of Fabricius excised at an early age for a previous, unrelated experiment were later used to obtain antibodies to *Salmonella typhimurium.* Injection of samples from a heated-inactivated broth containing the organisms resulted in an unexpectedly high death rate and very poor antibody production. Bruce Glick (1927–2009) and coworkers, recognizing the significance of their serendipitous observation, went on to show that bursectomy at 2 weeks of age profoundly decreased antibody production after a challenge with bacteria [113, 114]. This discovery ultimately led to establishing that lymphopoiesis in the bursa generated antibody-producing lymphocytes. Subsequent studies by Noel L. Warner (b. 1939) and Aleksander Szenberg, a Rockefeller Foundation Travelling Fellow from Poland and subsequently a staff member at the Walter and Eliza Hall Institute of Medical Research, Melbourne, confirmed the individual role of the bursa and the thymus in the immune system of fowl [115]. Thus, it was determined that the thymus and the bursa were primary lymphoid organs, so designated because they generated lymphocytes without the mediation of antigens. The bursa produced lymphocytes that could later make antibodies; these cells came to be referred to as bursa-derived or B lymphocytes. The early removal of the thymus impaired delayed hypersensitivity and permitted homografts (allografts) to be accepted. The thymus in chickens was discovered to produce lymphocytes that mediated cellular immunity and were referred to as thymus-derived or T lymphocytes. Poultry science proved to be of dramatic consequence for understanding mammalian immunology [116]. These findings were confirmed in mammals [117], and although the exact homologue of the bursa in mammals had been elusive, it was later considered to include the bone marrow, preserving the relevance of the designation "B" lymphocytes, purely by coincidence.

Observations in children with immunological diseases had confirmed that humoral and cellular immunity was regulated by separate determinants. In 1950, Eduard Glanzmann (1887–1959), professor of pediatrics at the University of Bern and later director of the Children's Hospital, and Paul Riniker, professor of pathology at Bern, described an infant who had markedly decreased antibody levels and decreased delayed hypersensitivity with absolute lymphocytopenia and a rudimentary thymus without lymphoid cells. This dual disorder became known as thymic alymphoplasia or Swiss-type agammaglobulinemia to distinguish it from the agammaglobulinemia of Bruton in which affected children had normal delayed immunity but deficient production of gamma globulin (antibody). In 1952, Colonel Ogden C. Bruton (1908–2003), Medical Corps, United States Army, serving at the Walter Reed Army Hospital described the first case of a human being with a predisposition to infections found to be the result of an inability to generate antibodies to infectious challenges. He determined the child had an absence of serum gamma globulin using a device that separated proteins based on their net electrical charge, an instrument and technique developed by Arne Wilhelm Kaurin Tiselius (1902–1971), a Swedish biochemist. Tiselius had trained with Theodor H. E. Svedberg (1884–1971), the inventor of the ultracentrifuge, for which Svedberg won the Nobel Prize in Chemistry in 1926. The Tiselius device permitted the separation of proteins in the fluid phase, an accomplishment that had a profound effect on protein chemistry and diagnosis in medicine. Tiselius was awarded the Nobel Prize in Chemistry in 1948 for his achievement. Tiselius went on to become President of the Nobel Foundation. The electrophoretic technique was advanced by adapting it to a solid phase, such as paper, cellulose acetate, or a gel. In 1966, Vincent Anthony Fulginiti (1931–2013), a pediatrician, then at the University of Arizona, and colleagues described

two sets of siblings who had thymic alymphoplasia but normal antibody synthesizing capacity. Thus, the effect of bursectomy in fowl or thymectomy in fowl or mice was recapitulated in humans by the effects of an inherited absence of the thymus or bursa-equivalent or both.

By 1969, the accumulated evidence for two principal types of integrated immune effector pathways, one mediated by B lymphocytes that mature into plasma cells to produce antibodies and one mediated by T lymphocytes derived from the thymus, was compelling and brilliantly synthesized by Ivan M. Roitt (b.1927) and colleagues [118]. Roitt and Deborah Doniach (1912–2004) had been the first to identify autoantibodies in Hashimoto thyroiditis and were convinced that many diseases were of an autoimmune nature. Ernest Witebsky (1901–1969), a German immigrant escaping the inhumanity of the Third Reich, and a giant in immunology, who settled in Buffalo, New York, working at the University of Buffalo School of Medicine, developed a model of experimental autoimmune thyroiditis. Roitt and Doniach and Witebsky and Noel Richard Rose (b. 1927), then a young colleague, are credited with simultaneously and independently, introducing the concept of autoimmune diseases. While in Germany, Witebsky had been influenced by the teachings of chemist and immunologist and Nobel Laureate in Physiology or Medicine, Paul Ehrlich. Ehrlich espoused the dictum of "horror autotoxicus," the necessity of the human immune system not to react to self-antigens. Witebsky was the student of Hans Sachs (1877–1945), professor of immunology and serology and at one point director of the Cancer Institute in Heidelberg, who had worked and published research with Ehrlich. The influence of Ehrlich's views slowed Witebsky's publication with Rose, currently professor at Johns Hopkins University, by 3 years until he was very sure that he was correct. Witebsky also defined autoimmunity more precisely than we do today. He insisted that there be demonstration of an autoantigen, not just an autoantibody, to be certain the process was autoimmune. Witebsky received the Karl Landsteiner Award in 1959 for his groundbreaking earlier work on blood group antigens.

Studies of human blood lymphocytes for the purpose of identifying immunoglobulin determinants on their surface using indicator red cells were being conducted by Robert Royston Amos (Robin) Coombs (1921–2006) and coworkers [119]. Coombs was renowned for his conceptualization and implementation of the red cell antiglobulin test in 1945. In later work, Coombs found, surprisingly, unsensitized sheep red cells formed rosettes with a large proportion of human blood lymphocytes. Coombs also found that a smaller population of lymphocytes carried membrane determinants for immunoglobulin or formed rosettes with complement-coated red cells [120]. The two populations of human blood lymphocytes were shown to have the phenotype of either thymocytes, i.e., were T lymphocytes, or immunoglobulin synthesizing cells, i.e., B lymphocytes [121, 122].

Inevitably, the cells of lymphoid malignancies began to be examined to determine their immunophenotype. It had already been deduced that myeloma was a B-lymphocyte malignancy and studies of Burkitt lymphoma cells in culture revealed their immunoglobulin-carrying phenotype, later to be denoted B lymphocytes [123]. An expansion of B lymphocytes with poorly expressed membrane immunoglobulin was found to be present in patients with chronic lymphocyte leukemia [121, 124]. Patients with other types of lymphocytic leukemia and lymphomas were soon characterized as to their T- or B-cell phenotype and, later, natural killer cell phenotypes, denoting the third of the three major lymphoid cell lineages.

In 1969, Joseph (József) Géza Sinkovics (b. 1924), a physician-scientist and Hungarian émigré, working in the Section of Clinical Tumor Virology and Immunology at the M.D. Anderson Cancer Center, observed that his unprimed lymphocytes killed allogeneic tumor cells in vitro. He reported his findings shortly thereafter [125]. He also showed that the cells involved in attaching to and lysing tumor cells were large granular lymphocytes. Although his report was met with skepticism and rejection (a project site-visitor proclaimed "There is no immune reaction without preimmunization"), this work was confirmed and expanded to indicate that the unprimed lymphocytes also could kill virus-infected cells. In his monograph [126], Sinkovics reviewed the reaction of the scientific community to his finding that tumor-reactive lymphoid cells might be present without preimmunization of their host. He provided the first photomicrographs and tumor cell growth-inhibitory graphs describing such cells.

This rejection of new ideas was commented on 400 years earlier by no less a figure than the French Renaissance author Michel Eyquem Montaigne (1533–1592) who remarked on the matter of discovery, "Whenever a new discovery is reported in the scientific world, they say first, 'it is probably not true.' Thereafter when the truth of the proposition has been demonstrated beyond question, they say, 'yes, it is true but it is not important.' Finally, when sufficient time has elapsed to fully evidence its importance, they say, 'yes, surely it is true and important, but it is no longer new.'" The population of lymphocytes carrying those capabilities was dubbed "natural killer cells," because their effect was neither mediated by antibody nor surface receptors nor did it require prior immunization. At about the same time (1970), Sinkovics described a lymphoproliferative disease, which in

retrospect may have had a natural killer cell phenotype [127]. Several investigators, notably Ronald B. Herberman (1940–2013), then at the National Cancer Institute, and from 1985 to 2009 the founding Director of the University of Pittsburgh Cancer Center, began to examine this matter and natural killer cells were determined to be a third type of lymphocyte [128]. With these advances, normal and pathological lymphocytes could be classified into distinct lineages (B, T, and natural killer [NK] cell types), which could be determined by expression of lineage-specific surface antigens and, eventually, by analysis of B-cell immunoglobulin and T-cell receptor genes [129, 130].

Application of Advances in Immunology to Classification

With advances in identification of lymphocyte subtypes, the rudimentary and partially erroneous classification of lymphoma could be expanded and made more accurate. Two such efforts stood out. In 1974, Harald Stein (b.1942), Edwin Kaiserling, and Karl Lennert (1921–2012), at the Patholisches Institut der Universität Kiel, established that reticulum cell sarcomas were not tumors of reticulum cells or histiocytes but were tumors of B lymphocytes by showing their cellular expression of immunoglobulin [131]. Karl Lennert was one of the leading histopathologists of the twentieth century interested in the classification of lymphoma. Lennert was born in Fürth, Bavaria, in 1921 and graduated from the medical school at the University of Erlangen in 1945. He studied at the Institute of Pathology at the University of Frankfurt and, thereafter, spent time at the University of Erlangen, the Max Planck Institute in Göttingen, the University of Frankfurt-am-main, and the Institute of Pathology at the University of Heidelberg. From 1963 to 1989, he was director of the Institute of Pathology at Kiel, during which years he and his colleagues developed one of the leading classifications of the lymphomas, referred to as the Kiel classification. The Kiel classification was the first to recognize B- and T-cell subtypes [107] (Table 37.3).

In a parallel second effort in the United States, Robert J. Lukes (1922–1994) and Robert Collins (1928–2013) developed a classification using immunological features of lymphoma cells [108]. Lukes' biography was commented on earlier because of his contribution to the classification of Hodgkin's disease. Robert Collins (b.1928–2013) was professor of pathology, at Vanderbilt University School of Medicine. Collins, along with Lukes, developed a system of categorization referred to as the Lukes and Collins classification, which denoted malignant lymphomas by their lineage of origin (B-cell or T-cell types). Collins and colleagues were also among the first to describe splenic and nodal marginal zone lymphomas, peripheral T-cell lymphomas, and anaplastic large-cell lymphomas. The Kiel classification used in Europe and the Lukes and Collins classification in North America were the two most prominent, widely used classifications of lymphoma in the 1980s and early 1990s (Table 37.3).

Hematologists, oncologists, and experimental pharmacologists needed to interpret results of clinical drug trials performed in different institutions, worldwide, a situation made difficult by the use of different classification schemes that did not easily permit equivalencies among pathologists. The problem was addressed initially by the United States National Cancer Institute, which convened a large group of investigators to determine whether one classification scheme was better than another at predicting clinical outcome. None of the classifications predicted patient outcome better than another. Therefore, a "Working Formulation" was developed so that physicians could translate clinical data derived in different institutions, using different classification schemes [109]. In the latter Formulation, lymphomas were divided into ten categories based on morphological features, permitting physicians to deal with a large number of lymphoma subtypes. The lymphomas were further grouped into three clinical prognostic groups (aggressive, intermediate, and indolent). Although the Working Formulation was not intended to be a final classification scheme, it was used as such by many institutions, particularly in North America. However, the Working Formulation failed to distinguish the distinctive features of several newly described histological subtypes of lymphoma. Moreover, the Working Formulation categories did not include newly available immunological and molecular genetic analyses capable of distinguishing new subtypes of lymphomas, such as mantle cell, marginal zone, and peripheral T-cell lymphomas [110].

A new approach to lymphoma classification proposed by the International Lymphoma Study Group used clinical features, morphology, immunophenotype, and genetic alterations to define the unique lymphomas that could be diagnosed consistently by hematopathologists. This grouping, published in 1994, was known as the Revised European-American lymphoma (REAL) classification [111]. The cell lineage of origin of each lymphomas was defined as well [132] (Table 37.3).

In the late 1990s, a new World Health Organization (WHO) classification for clonal lymphocytic disorders was being developed, based on the REAL classification. First published in 2001, updated in 2008, and again in 2016, the WHO classification represented the consensus of an international group of hematopathologists, including contributions from a clinical advisory committee of hematologists and oncologists experienced in treating lymphomas [112]. This array of over 50 distinct lymphoma histopathological types derived from transformation of B, T, and NK progenitor cells has become the accepted classification, worldwide (Table 37.3).

Application of Technological Advances in Immunology and DNA Analysis to Classification

The progress in the identification of the lymphoma subtypes was dependent on several critical technical advances of importance. The process for developing monoclonal antibodies was one of the most important. César Milstein (1927–2002), a biochemist and émigré from Buenos Aires, working in Medical Research Council Laboratory of Molecular Biology in Cambridge, England, was studying methods of tumor cell fusion. Milstein's research career was devoted to studying the structure of antibodies and the mechanism by which antibody diversity is generated. Georges Jean Franz Köhler (1946–1995) working in Basel, Switzerland, was studying normal antibody-producing cells in tissue culture. His research was impaired as he could only get small quantities of cells to survive for short periods of time. Köhler, a young scientist, knew of Milstein work, and it seemed logical to see if normal antibody-forming cells could be fused with myeloma cells to produce long-lived hybrid cell lines that produce antibody. Köhler went to Milstein's laboratory as a postdoctoral fellow, and they solved the problem in 2 years, 1975–1976. They developed a method of lymphocyte fusion that provided a tool to overcome these limitations. Mouse myeloma tumor cells were fused to spleen cells derived from a mouse, which previously had been immunized with antigen. About 50% of the hybrid cells combined desired parental traits: vigorous growth in tissue culture derived from the myeloma tumor cell and antibody production coming from the immunized splenic B cells. Köhler and Milstein called these conjoined cells "hybridomas." A relatively high proportion of the hybridoma cells secreted antibody specific to the immunizing antigen. The technique provided specificity, since each hybrid produced only one antibody and an unlimited supply of antibody, since the hybrids were "immortal," growing as tumor cells. Also, impure antigens led to pure antibody reagents since the hybrids produced one principal antibody. The monoclonal antibody by definition characterizes only one antigen of the many injected into the mouse. Milstein and Köhler [and Niels K Jerne (1911–1994)] were jointly awarded the Nobel Prize in Physiology or Medicine in 1984 for their achievement. Milstein made many contributions to the improvement of monoclonal antibody technology, especially focusing on their use as markers that allow distinction among different cell types. He also foresaw the potential of ligand-binding reagents that could result from applying recombinant DNA technology to monoclonal antibodies and inspired the development of the field of antibody engineering.

Wallace Coulter's (1913–1998) development of the Coulter counter and the use of the Coulter principle on which it depended to develop the first particle (e.g., single cell) flow analyzer was an important contribution to science and medicine. The development of the electronic particle counter and flow cytometry and particle sorting has had a stunning and incalculable impact on research, diagnosis, and therapy. Adaptations that permit characterizing the phenotype or DNA content of cells, and sorting and isolating cells by their physical or surface properties have advanced studies of cells, dramatically. This remarkable instrument evolved from Coulter's earliest developments based on his idea that particles moving through a small aperture across which a current flowed would produce an impedance that could be recognized electronically. Moreover, the height of the signal could be made proportional to the volume of the particle. This, the Coulter principle, resulted in cell counters that were integral to medical research and diagnosis, and to industrial uses. A small subsidiary company owned by Coulter, Los Alamos Particle, Incorporated, in collaboration with the Health Physics Division of the Los Alamos National Laboratories used the ideas that Coulter had evolved to develop to count and size particles for the prototype flow cytometer. Mack Jett Fulwyler (1936–2001) and colleagues [133, 134] reported on the instrument they developed that used the Coulter principle to separate fluid droplets containing single cells by differences in cell volume. They could electrically charge droplets with cells of the desired volume and deflect them electrostatically into a collecting reservoir. A group of engineers and scientists, working at Stanford, advanced this technology by using cells stained with fluorescent dyes and measured cell size by light scatter and the fluorescence intensity generated by a laser [135]. The cell-containing droplets could be charged based on size and on the conversion of the laser-generated optical signal into an electronic pulse. These two variables (dual parameter) could be used to decide whether to deflect the cell into a collecting reservoir. The addition of laser light scatter to the Coulter principle of electronic counting and sizing resulted in VCS technology (**v**olume, **c**onductivity, **s**catter of laser light). The instrument, called a fluorescence-activated cell sorter (FACS), was invented in the late 1960s by Leonard A. Herzenberg (1931–2013), with the contribution of several colleagues, to do flow cytometry and cell sorting of viable cells [136]. Becton Dickinson Immunocytometry Systems introduced the commercial machines in the early 1970s, using the Stanford patent and expertise supplied by the Herzenberg laboratory and a Becton Dickinson engineering group under Bernard Shoor. Herzenberg won the Kyoto Prize in 2005 for his technological contribution to science and medicine. Wallace Coulter's ideas and the instruments they spawned were among the most important innovations in diagnostic and investigative hematology, immunology, and cell biology, among other disciplines, in the twentieth century.

The availability of monoclonal antibodies and flow cytometry led to advances in the classification and diagnosis

of clonal lymphoid diseases and, ultimately, to their therapy with monoclonal antibodies directed against cell surface proteins identified by cell flow analysis (discussion later in this chapter).

A third technological advance was made to further subclassify lymphomas by molecular differences. Development of techniques to array and identify the expression of a specific cell's genes on a microchip has permitted characterization of all or a subset of genes in a specific normal or neoplastic lymphocyte cell type, based on the pattern of its genic expression, overexpression, or under-expression. Using DNA microarray technology, the messenger RNA levels of the entire genome could be quantified in a single experiment. Louis Michael Staudt (b. 1955), at the National Cancer Institute, a pioneer in the use of this technology, developed the Lymphochip, a specialized array of 18,500 complementary DNAs containing human genes important in lymphocyte biology and the normal and pathological immune response [137]. Staudt, together with Arash A. Alizadeh (b.1972) and other colleagues, discovered that a pattern of gene expression within a homogeneous histopathological and immunological phenotype, diffuse large B-cell lymphoma, was heterogeneous with three subtypes by DNA microarray analysis of which two were indicative of the cell type of origin of the lymphoma [138]. Diffuse large B-cell lymphoma biopsy samples could be stratified into at least two gene expression subgroups based on the data obtained from complementary DNA microarrays. The germinal center B-cell-like subgroup expressed genes characteristic of normal germinal center B cells, whereas the activated B-cell-like subgroup expressed a subset of the genes that are characteristic of mitogenically activated blood B cells and other genes characteristic of plasma cells, particularly those encoding endoplasmic reticulum and Golgi zone proteins involved in secretion. The subgroups identified had significantly and dramatically different 5-year survival rates after identical multiagent chemotherapy. The germinal center B-cell-like subgroup had twice the proportion of 5-year survivors than did the activated B-cell-like subgroup. Thus, gene expression profiling could subclassify diffuse large B-cell lymphoma into biologically and clinically distinct groups. This approach has found important variations of prognostic significance within a single diagnostic category in other lymphomas and has identified molecular pathways that are targets for future drug development.

The emergence of DNA microarray technology and the gene chip involved the genius of several men and numerous academic and industry consultants [139]. Alejandro Zaffaroni (1923–2014), born, raised, and educated in Montevideo, Uruguay, came to the United States to obtain his Ph.D. in biochemistry at the University of Rochester School of Medicine. After receiving his degree, he joined the Syntex Corporation in Mexico City in 1951, then a small Mexican chemical company specializing in steroid research. Over the next 2 decades, Zaffaroni became its Chief Executive Officer and transformed Syntex into a major multinational pharmaceutical company based in Palo Alto, California. He was one of the most innovative scientists and business men of his age and focused on the areas of drug delivery and drug discovery. He left Syntex to start ALZA Corporation (named for the first two letters of his first and last name) to advance his work on transcutaneous ("the patch") drug delivery and later he established the DNAX company, among other ventures. In 1988, he started Affymax, recruiting outstanding scientists from Stanford and other universities to develop singular and innovative means of developing new drugs. They recruited a gifted young chemist Stephen Philip Allan Fodor (b. 1953) who received his Ph.D. in chemistry from Princeton. At the end of his postdoctoral fellowship at the University of California at Berkeley, Fodor was convinced to leave academia because of the brain trust recruited by Affymax. Fodor was attracted to the brilliance of the persons involved and the ideas to be pursued. They began by using semiconductor chip methodology and adapting it to generating peptide diversity on an array using a photolithographic process. Eventually, Fodor and the team turned from peptide microarrays to nucleic acid microarrays. The process of making the chip was perfected by Fodor and his team. First, photolithography was used to create a huge number of molecules on a silicon wafer or a glass surface. A single chip measuring 1.28 cm × 1.28 cm can hold more than 400,000 probe molecules. Next, a target molecule is labeled with fluorescent dye and is simultaneously hybridized to all the probes on the chip. A fluorescent image develops that is scanned by a laser, producing data for computer analysis. The intensity of the fluorescent light generated in this process depends on how avidly the target molecule hybridizes with the probe molecule attached at each position on the chip. All this began as a complex experimental procedure and eventually became fully automated. Patrick O. Brown (b.1954), professor of biochemistry at Stanford University, independently had developed microchip technology using complementary DNA rather than oligonucleotides, a useful and important advance in the methodology for the study of the human genome and that of lower species.

Fodor and Zaffaroni co-launched Affymetrix in 1991, a spin-off of Affymax, and developed the chip technology to identify large arrays of genes, permitting Staudt and others to apply these methods to studies of lymphoma diagnosis by genic rather than phenotypic characteristics and to explore the pathobiology of lymphoma by analyzing their gene expression patterns.

Perhaps, one of the most powerful and important techniques that can be applied to the diagnosis or management of lymphoma is the polymerase chain reaction The story of the antecedents to the development of the polymerase chain reaction is not detailed here, but can be found in "A Short

History of the Polymerase Chain Reaction" [140]. The Nobel Prize in Chemistry was awarded to Kary Bank Mullis (b.1944) for the idea and pursuit of the development of this momentous technology. It has been a profound contribution to the study of DNA molecular biology (e.g., sequencing), the diagnosis of inherited disorders, infectious diseases, various cancers, and to forensic sciences, among other disciplines. Mullis, a DNA chemist at Cetus Corporation in the early 1980s, had an epiphany about a method to amplify a small segment of DNA to millions of identical copies in just a few hours sufficient for analysis. The idea was met by disdain by his colleagues and supervisors at Cetus, initially. His recollections about the idea and its pursuit are described in his Nobel lecture [141]. This method allows clinicians to diagnose and monitor diseases using a minimal amount of blood or tissue sample. The detection of monoclonal expansions of the immunoglobulin heavy chain (IgH), the T-cell receptor-gamma (TCRgamma) chain, or the *BCL2* genes, for example, has become an important addition to the methods to diagnosis and manage patients with lymphomas.

Recognition of Cutaneous Lymphoma

Anne Charles Lorry (1768–1837) published, perhaps, the first textbook on skin diseases in which he described multiple and festering skin lesions he described as "fleischgewächse" (sarcome). It is not clear whether these lesions represented mycosis fungoides. Mycosis fungoides, as we know it today, was described by the French dermatologist Jean-Louis-Marc Alibert (1768–1837) working at l'hôpital Saint-Louis in Paris. He initially called the lesions pian fungoides in 1814 because of their similarity to Yaws (pian ruboide). In 1832, the year Hodgkin reported his seven cases of lymphadenopathy, Alibert described the skin tumors of this severe case as "a strange disorder of the skin with mushroom-like tumors"; hence, his use of the term mycosis fungoides in his treatise on skin pathology, Monographie des Dermatoses [142]. He knew it was not caused by a fungus. The patient, Lucas, began with a desquamating rash (éruption furfuracée) and it evolved into numerous facial and truncal tumors and death 5 years later (Fig. 37.4). Given the times, Alibert had no idea of the underlying pathology. In 1864 Heinrich Köbner (1838–1904) published one of the earliest descriptions of its histopathological appearance. He thought it a granulomatus disease. In 1869, Xavier Gillot (1842–1910) and Louis-Antoine Ranvier (1835–1922) connected mycosis fungoides with a proliferation of lymphocytes, referring to it as a cutaneous manifestation of lymphoma (lymphadénie cutanée). The former was a student working for his doctorate in medicine under Ranvier. Ranvier was one of the leading neuropathologists of that period. He published the first edition of his "*Manuel d'histologie*

Fig. 37.4 Alibert's patient with lesions he called "mycosis fungoides." The patient, Lucas, was pictured in the second volume of Alibert's treatise *Monographie des Dermatoses* in 1832. The disease was first manifest as a desquamating rash and progressed to the multiple lesions shown on the patient's face and trunk (Source: Jean Louis Marc Alibert's Monographie des Dermatoses. Paris, 1832)

pathologique"("*Manual of Pathological Histology*") in 1869 and "*Traité technique d'histologie*" ("*Treatise on Histological Techniques*") in 1875, the former based on private histopathology lessons he and a colleague offered to students in Paris. In his thesis, Gillot also described four stages of cutaneous lymphoma, starting with the erythematous stage and ending in the tumoral stage with ulcerations and hardening. Pierre-Antoine-Ernest Bazin (1807–1878) gave a comprehensive description of mycosis fungoides in 1870, 1 year after Gillot, describing the disease as having three stages: nonspecific erythematous (premycotic), plaque (lichenoid), and tumor (fungoid) phases. He saw these as a progression of one phase into another over an extended period of time. Subsequently, it was appreciated that the disease could start with a tumor stage without an earlier erythematous phase. For a time, the disease was referred to as Alibert-Bazin disease. Its relationship to lymphoma and its neoplastic, rather than inflammatory, nature was further highlighted by John Frank Fraser, a dermatologist at Cornell Medical School in 1925 [143]. In 1938, Albert Franz Sézary (1880–1956), a dermatologist who was born in Algiers but later studied and practiced in Paris, collaborating with Yves

Bouvrain (1910–2002), described a disease in which erythroderma was associated with unusual cells in the blood ("… cellules monstreuses dans le derme et le sang circulant") [144]. Sézary thought the cells were monocytes or histiocytes. In 1929, Sézary became chef de service at les hôpitals Broca and Saint-Louis. He was president of Société française de dermatologie in 1937 and was elected to l'Académie de Médecine in 1945. He was also Officier de la Légion d'Honneur. Bouvrain went on to be a noted student of cardiac rhythm disturbances and an expert in cardiac electrophysiology. In 1971, Peter E. Crossen and colleagues provided evidence that the cells in Sézary syndrome were lymphocytes, and in 1973, Jean-Claude Brouet, a French immunohematologist, and coworkers reported that the malignant lymphocytes in Sézary syndrome carry immunological features of T lymphocytes [145]. Marvin A. Lutzner, chief of the Dermatology Branch at the National Cancer Institute, had studied the ultrastructural and clinical features of these diseases and described the cerebriform nuclear shape of the cells in the blood in patients with Sézary syndrome. He recognized the closely related features of mycosis fungoides, Sézary syndrome, and psoriasis en plaque and in 1975 at a National Institutes of Health conference, he and his coworkers proposed that these disorders be grouped under the designation of cutaneous T-cell lymphomas [146].

Therapy of Lymphoma

By the early 1940s extensive series of cases of lymphoma had been published and awareness of the extranodal sites that could be involved, e.g., bone, gastrointestinal tract, genitourinary tract, and others, had become evident. In addition, the appreciation that a large proportion of cases had extranodal involvement or may be limited to extranodal sites was by then apparent. In 1940, Everett D. Sugarbaker (1910–2001) and Lloyd Freeman Craver (b.1892) had published a review of lymphosarcoma in the *Journal of the American Medical Association*. Maxwell M. Wintrobe, the most distinguished and accomplished hematologist of the first half of the twentieth century, had published the first edition of his now classic textbook *Clinical Hematology* in 1942 in which he reviewed the knowledge of lymphoma up to that time and described the multiple organ involvement that could ensue. The terminology of the lymphoma subtypes was largely that described by Gall and Mallory. Wintrobe classified treatment into three categories: Fowler's solution (solution potassium arsenate, U.S.P.), surgery (describing extended survival in a small proportion of patients with lymphoma and Hodgkin's disease after excision of nodes), and irradiation with X-rays or radium. An extensive discussion was provided about the X-ray energy levels favored, the duration of treatment, and the approach, e.g., localized versus generalized radiation. The treatment advances in the lymphomas were based on the advances made for the treatment of Hodgkin's disease. Initially, in the first half of the twentieth century, radiation therapy was used principally on localized lymphomas or used on selected troublesome areas for palliative therapy. Since lymphomas were (1) usually widely dispersed, even if not apparent by the diagnostic techniques of the day, (2) often had neighboring occult disease outside the radiation fields, and (3) were being treated with X-ray machines delivering orthovoltage radiation, an inadequate technique, radiation effects were usually short-lived.

In the 1950s and early 1960s, single-agent chemotherapy (nitrogen mustard or newer alkylating agents, methotrexate, vinca alkaloids, and glucocorticoids) or combination (two or three) agents provided more therapeutic approaches for lymphoma. It was the evolution to combinations of four or more drugs, in the 1970s, developed on the model of MOPP therapy for Hodgkin's disease that led to sustained remissions and cures of some aggressive lymphoma subtypes with drugs [147].

In the late 1990s, the introduction of monoclonal antibodies designed to target specific cluster of differentiation antigens (proteins) on the lymphocyte surface further enhanced therapy. In 1980, Philip Paul Stashenko (b. 1947) and colleagues described a B-lymphocyte marker (antigen), B1. In 1983, Lee Marshall Nadler (b.1947) and colleagues reported that a monoclonal antibody to B1 reacted with B-cell lymphomas and non-T-cell acute lymphoblastic leukemias. The B-lymphocyte antigen, B1, had been designated CD20, and was subsequently characterized and cloned. By the mid-1980s, studies of monoclonal antibodies for the treatment of lymphoma had been conceived and phase I and II trials were underway. After much study, in 1997, rituximab, a recombinant, chimeric anti-CD20 molecule, was the first monoclonal antibody approved for the treatment of follicular, CD20-positive, B-cell lymphoma; in 2006, it was approved for initial therapy of diffuse large B-cell lymphoma in combination with drug regimens containing an anthracycline antibiotic [148]. The addition of rituximab to multidrug chemotherapy regimens has enhanced the rate of and duration of remission in the lymphoma subtypes for which it is used. The development of techniques to chimerize mouse variable regions of antibodies or those of other species with the constant region of human immunoglobulin by a team of scientists from Columbia and Stanford Universities and Becton-Dickinson Company made these approaches practical [149]. A chimeric antibody is one in which the variable domains (antigen-binding sites) are from the species used for immunization (e.g., mouse) and the constant domains are protein chains derived from human isotypes. This chimerization with a human constant region reduces substantially the possibility of an immune response by the patient against the xenogenic portion of the protein [150]. In this case, the variable domains

are derived from the murine monoclonal antibody generated against CD20. The history of the commercial development of rituximab is detailed in the chapter, The Discovery of Rituxan, in the monograph *New Drug Discovery and Development* [151].

Discovery of Virus- and Bacteria-Related Lymphomas

Burkitt Lymphoma (Né African Lymphoma) and the Epstein–Barr Virus

In 1946, Denis Parsons Burkitt (1911–1993), after obtaining a degree as Doctor of Medicine from Trinity College, Dublin, and his surgical training at the University of Edinburgh, which he completed in 1938, took a position as a surgeon in the British Colonial Service in Uganda. His uncle, Roland Wilks Burkitt (1872–1946), was the first practicing surgeon in East Africa (Kenya). This family relationship and his desire to work with a mission resulted in Burkitt leaving studies in engineering, his father's profession, and pursuing medicine. Initially, he was refused an opportunity to work as a surgeon in Africa because he had lost one eye in a childhood fight on his way to the Protestant school he attended in Enniskillen at a time when the city was plagued by religious strife (now Northern Ireland). The onset of World War II resulted in his joining the Royal Army Medical Corps in 1941; he was initially stationed in England. From 1943 to 1945, ironically, he was posted to the British Colonial African Service in Kenya and Somaliland. After separation from military service, having proven his capabilities, despite having vision in one eye, he was accepted into the British Colonial Service in Uganda in 1946. He began his service in Lira, Lango District, Uganda, where he was placed in charge of the medical services of several hundred thousand persons with ill-equipped and primitive hospital facilities, essentially a 100 bed "bush hospital." There he provided medical and surgical services to the native inhabitants. He, in parallel, did some clinical research and published a paper on the high frequency of hydrocele in the eastern, but not the western, part of the District, which later proved to be the result of filariasis in the east. These experiences developed his interest for geographical medicine and epidemiology. He, thereafter, was transferred to Mulago Hospital of the Makerere College just outside Kampala. During the next decade of medical practice in that region, Burkitt saw with surprising frequency a rapidly progressive, destructive tumor of the jaws in African children. These tumors had rarely been reported and, in large part, they had been misclassified as soft tissue sarcomas, retinoblastoma if in the orbit, neuroblastoma if in the adrenals, or granulose cell tumors if in the ovaries.

Jaw tumors in Ugandan children had been observed in the first decade of the twentieth century and round cell sarcomas of the jaw and ovary were reported from Nigeria, French West Africa, the Belgian Congo, and the Cameroons. R.A. Hoyte and F.M. Simmonds practicing near Accra in the 1920s treated jaw tumors in children with a polypharmaceutical product made in France called Septicemine. The principal active ingredient of the concoction was hexamine and was used for the treatment of yaws, gonorrhea, typhus, and other infectious diseases in Africa. When Hoyte and Simmonds observed responses, they concluded the tumor was probably infectious in origin. Forty-years later, Burkitt, who learned of their observations, showed that the drug had transient efficacy and that Hoyte and Simmonds surely were treating the lymphoma that now bears Burkitt's name.

In 1957, the physician in charge of the pediatric ward at Mulago Hospital, Hugh Carey Trowell (1904–1989), asked Burkitt to see a 5-year-old child with masses involving both sides of the upper and lower jaws. The biopsy was interpreted as a granuloma. A few weeks later Burkitt was visiting a colleague at Jijoya hospital and saw a child in the courtyard with the same clinical features. He examined the child and found a coincidental abdominal mass. He concluded that one such case was a curiosity but not two. He decided to do a systematic inquiry into this unexplained disorder. On his return, he started by doing extensive record reviews of African children with tumors at Mulago Hospital and realized that the facial and abdominal tumors were linked and probably represented one disease. He published his classical description of jaw tumors in African children in 1958 [152]. Burkitt initially did not recognize the lymphoid origin of these tumors, but he deduced that the disease was a single entity, even when it was disseminated to ovaries, adrenals, heart, stomach, liver, pancreas, and the central nervous system, as it sometimes presented. He enlisted his colleagues in the Department of Pathology, Gregory Thomas O'Conor (1929–2012) and Jack Neville Phillip Davies (1915–1998), to review, retrospectively, the surgical biopsies from these cases and they found them to be identical and a type of lymphoma [153].

As Burkitt was publishing his first paper on the subject, he contacted Alfred George Oettlé (1919–1967), director of the Cancer Research Unit of the South African Institute for Medical Research, who was experienced in the variations in cancer expression. Oettlé responded that the tumor does not occur in South Africa! Prompted by this unexpected response, Burkitt set out to trace its distribution, first within the territory of his posting, and, subsequently, throughout the African continent. He traveled with two physician colleagues throughout Uganda and East and Southern Africa in a used Ford stationwagon covering about 16,000 km and visiting 60 hospitals. He, also, sent illustrated letters to the government and mission clinics on the continent, estimated to be nearly

1000 health facilities [154]. The study was supported by a grant of 250 pounds from the Medical Research Council of the United Kingdom and about 150 pounds from other agencies, some of which paid for the Ford wagon. This relatively inexpensive, homespun, but ingenious approach to population research led to the demonstration of a well-circumscribed area of prevalence, including not only Uganda, but also other central African countries and territories: Kenya, Tanganyika, Nigeria, and the Congo. All of these regions had tropical climates. By contrast the disease was not observed in South Africa or in Sudan or other semiarid or arid regions. Burkitt had mapped out the area of incidence of what he called "African lymphoma." Alexander John Haddow (1915–1978), Director of the East African Research Institute at Entebbe, noted that the altitude distribution was an index of temperature barrier of about 60 °F and areas of low rainfall defining a rainfall barrier. Haddow suggested to him that the geographical distribution of the disease, only in tropical areas, corresponded to the distribution of the tsetse fly and yellow fever, suggesting an arthropod-borne etiology (Fig. 37.5). An outbreak of o'nyong nyong fever in the African lymphoma belt in 1959, caused by a mosquito-transmitted alphavirus, added to the argument. This means of transmission proved not to be the case but it provoked a search for an infectious etiology.

Burkitt and Michael Anthony (Tony) Epstein (b.1921) met on March 22, 1961, in London during a lecture by Burkitt entitled "The commonest children's cancer in Tropical Africa: a hitherto unrecognized syndrome." [155] In the talk, delivered at the Middlesex Hospital in his enthusiastic style, Burkitt described the distribution of cases that he mapped out in his studies in Africa and postulated that there may be an infectious component to African lymphoma. Few in the small crowd at the lecture were excited by this proposition, but one attendee, Tony Epstein, a pathologist and tumor virologist working on Rous sarcoma virus in the Bland Sutton Institute of the Middlesex Hospital Medical School, was excited. This tumor sounded to him as if it could have a viral etiology. After the presentation, the two men met and Burkitt agreed to send Epstein frozen tumor specimens for him to analyze. Epstein's efforts to isolate a virus from these samples or to identify viral particles in transmission electron micrographs of biopsy tissue over several years were a failure. In 1964, Burkitt sent another tumor specimen to Epstein's laboratory. The flight, the overnight BOAC Comet flight from Kampala to Heathrow, was diverted to Manchester because of fog. By the time the specimen arrived in Epstein's laboratory it was cloudy, usually a sign of bacterial contamination. On closer inspection of a wet preparation no bacteria were seen. Epstein realized the cloudiness was the result of

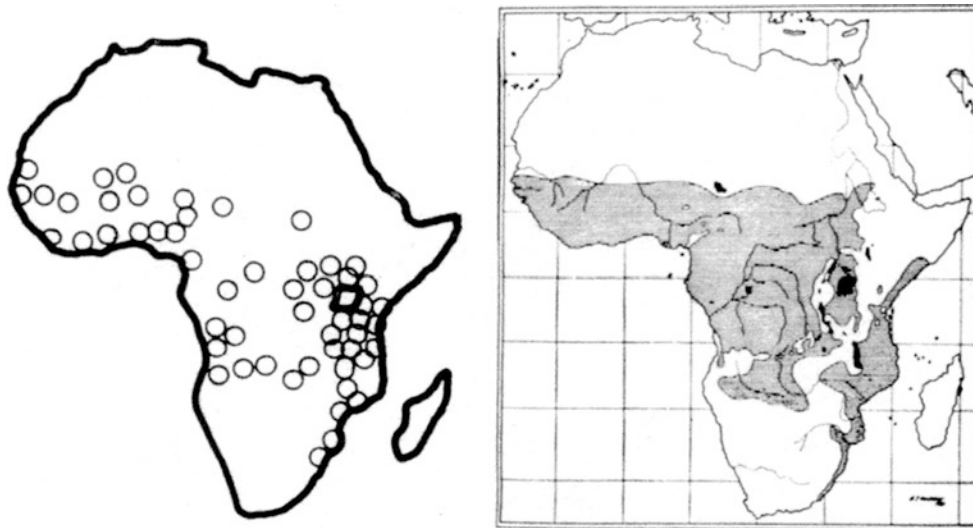

Fig. 37.5 Maps shown by Burkitt in his lecture delivered to the Royal College of Surgery on May 24, 1961. The map on the *left* indicates the localities in which Burkitt found cases of African lymphoma during his epidemiological safari through Africa or by correspondence with area hospitals. On the *right* is a map of Africa, prepared by Haddow showing in *white* the areas either over 5000 ft. elevation, where the seasonal mean temperature may fall to less than 60 °F, or areas with less than 20 in. of rainfall per year. Burkitt *highlighted* the concordance of the *shaded areas* in the map on the *right* with the areas with cases of African lymphoma on the *left*. Burkitt also was impressed with the similarity of tissue sites involved in polyoma virus-infected hamsters and African lymphoma. He got the latter idea from Davies working in the department of pathology at Makerere College Medical School, who proposed African lymphoma might be a virally induced disease. The lecture sponsored by the Royal College of Surgery had sparse attendance, such was the lack of interest in these findings at the time. It was at an earlier presentation of this lecture in March 1961 at Middlesex Hospital that Burkitt met Anthony Epstein and they solidified their future collaboration, leading to identification of the viral particles in the cell line of a patient with African lymphoma (Copyright The Royal College of Surgeons of England; reproduced with permission. Reproduced in O'Conor GT, Davies JNP. Malignant tumors in African children. With special reference to malignant lymphoma. J Pediatr. 1960;56:526–35)

sloughed tumor cells growing exponentially. He developed the first of several continuously growing cell lines (EB1) from that patient's sample and, when he examined the cells by electron microscopy, recognized they contained viral particles (Fig. 37.6). At that time, Uganda was a peaceful and prosperous country, referred to as The Pearl of Africa. Several years later it fell into the hands of Idi Amin Dada (1925–2003), a ruthless tyrant, after which time this remarkable collaboration and discovery might not have happened. The team of Epstein, Bert Geoffrey Achong (1928–1996), and Yvonne Barr (b.1932) published their dramatic results in 1964 [156]. The inability to grow the virus in tissue culture and the skepticism about electron microscopy as a means to discover viruses resulted in delay in broad acceptance of these findings. Eventually, further work made the findings irrefutable. The virus, which came to be known as the Epstein–Barr virus (EBV) and is classified as human herpesvirus-4 (HHV-4), is found in the cells of nearly all cases of the African or the endemic form of Burkitt lymphoma. Achong's training in preparing tissues for electron microscopy was a technical contribution to Epstein's research team, which included, Barr, a Ph.D. graduate student when she first joined the laboratory in the Sutton-Bland Pathology Institute on the first floor of Middlesex Hospital. Barr an Australian, later returned to that country. Ashong was able to produce electron micrographs of the lymphoma cells growing in culture,

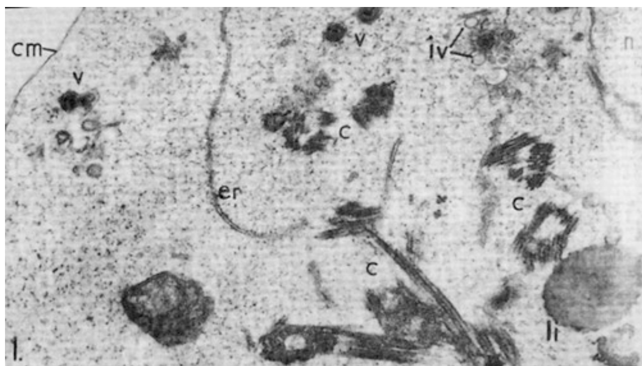

Fig. 37.6 The first visualization of viral particles in a transmission electron micrograph of a cell from a cell line of a patient with Burkitt (African) lymphoma. Epstein and Barr were unsuccessful in culturing the virus over several years of trying "a fruitless struggle," Epstein declared, but found it in his first micrograph of the cell-line developed from the tumor specimen sent to him by Burkitt. As soon as he examined the micrograph he knew he had viral particles. *cm* cell membrane; *n* nucleus; *v* mature viral particles; *iv* immature viral particles; *c* crystals cut in various planes; *li* lipid body; *er* endoplasmic reticulum; numerous free ribosomes in cytoplasm. Yvonne Barr Balding in a Youtube video in 2014 sending greetings to the participants at a conference in Oxford on the 50th anniversary of the discovery, recalls the Lancet calling their office asking if the paper was ready. The Journal was anxious to publish this extraordinary finding, perhaps evidence of the viral etiology of a lymphoma. (Reprinted with permission of Elsevier from Epstein MA, Achong BG, Barr Y. Virus particles in cultured lymphocytes from Burkitt's lymphoma. Lancet. 1964; 1(7335):702–3)

in which Epstein immediately recognized virus particles. The virus, not coincidentally, confers immortality on B cells in culture and was subsequently found to be the virus that causes infectious mononucleosis and is strongly associated with at least three other malignancies: Hodgkin lymphoma, lymphoma in immunodeficient individuals, and nasopharyngeal carcinoma. However, whereas the Epstein–Barr virus was isolated from almost all African cases, it was identified in only a fraction (~20%) of the biopsies of nonendemic cases that share the same cytogenetic translocations but differ in clinical behavior and slightly in molecular genotype. This virus has been identified in lymphomas that complicate a variety of inherited and acquired immunodeficiency states: ataxia-telangiectasia, Wiskott-Aldrich syndrome, severe combined and common variable immunodeficiencies, the X-linked immunodeficiency syndrome, and lymphoma associated with the immunosuppression of organ transplantation and of the acquired immunodeficiency syndrome, notably those with immunoblastic or primary central nervous system lymphomas. In all these conditions, the virus is clonal, but its role as a causal factor of the lymphoma, if any, remains to be elucidated.

At a conference sponsored by the Union Against Cancer in 1963 African lymphoma became commonly called "Burkitt's tumor" and, subsequently, "Burkitt's lymphoma."

In 1966, Burkitt returned to England and took a position with the Medical Research Council. He met a naval surgeon Thomas Latimer (Peter) Cleave (1906–1983), an insightful nutritionist, who had ideas about the relationship of dietary patterns in the population and certain diseases of developed countries. Together they studied and wrote about the importance of dietary fiber [157]. Burkitt did epidemiological studies on diseases in the developed world and their relationship to dietary fiber. In 1975, Burkitt coauthored a book entitled "*Refined carbohydrate foods and disease: some implications of dietary fiber*" with Hugh Trowell and, 10 years later, *Dietary Fibre, Fibre-Depleted Foods and Disease.* Trowell, his former colleague in Uganda, had first asked him to see a child with jaw masses, the case that kindled Burkitt's interest in the disease. Burkitt also published a book for popular consumption "*Don't forget fiber in your diet.*" Burkitt had made "stool rounds" strolling through the African bush, taking photographs of human feces. He found that Africans produce several times the amount of soft feces as Western people. He reasoned that we evolved to live off the land on vegetables and commented on the rarity in Africa of gallstones, diverticulitis, appendicitis, obesity and its sequelae, and several other diseases common in the West. He said that the health of a country's people could be better determined by the size of their stools and whether they floated or sunk than by the country's technological advancement. In 1971, he published a classic article in the journal *Cancer* on the relationship of dietary fiber to colon cancer.

Burkitt's tumor was characterized immunologically as a B-cell lymphoma. In 1972, two Bulgarian scientists, Georgi Manolov (1927–2004) and Yanka Manolòva (b.1928), a husband and wife team working at the Karolinska Institute in Stockholm, reported that there was additional chromosomal material on chromosome 14 (14q+) in the cells of Burkitt lymphoma [158]. This important finding initiated a cascade of additional genetic discoveries regarding Burkitt lymphoma cells. In 1969, Lore Zech (1924–2013), also working at the Karolinska Institute in the laboratory of Torbjörn Oskar Caspersson (1910–1997), had found that a stain, quinacrine mustard, resulted in chromosomes developing light and dark transverse bands along their length. This banding method permitted cytogeneticists to identify accurately each of the 22 autosomes and the X and Y chromosomes and to determine the region (band) at which a chromosome break occurs. Ultimately, Giemsa staining became the most utilized method of chromosome identification and banding. In 1976, Zech and coworkers proved that the extra material on chromosome 14 was the result of an unbalanced translocation between chromosomes 8 and 14 (t(8;l4)(q24;q32)) [159]. Subsequent investigation demonstrated that the translocation was present in approximately 80% of Burkitt lymphoma cell lines; the remaining 20% revealed one of two variant translocations between chromosome 8 and either chromosome 2 (t(2;8)(p12;q24)) or chromosome 22 (t(8;22)(q24;ql 1)). In 1982, both Philip Leder (b. 1934) and Carlo M. Croce (b. 1944) and their associates cloned the translocation breakpoint, and identified the *MYC* oncogene at the site of the break at band 24 on the long arm of chromosome 8 (8q24) [160, 161]. Transfer of *MYC* coupled to the immunoglobulin heavy chain or light chain genes by injection into the germline via the egg nucleus resulted in aggressive B-cell lymphomas in experimental animals, but complete or truncated *MYC* alone was inactive.

Thus, African Burkitt lymphoma cells were invariably found to have one of three chromosomal translocations, each involving the *MYC* proto-oncogene on chromosome 8 and the gene for the immunoglobulin heavy chain or the kappa or lambda light chain on chromosome 14, 2, and 22, [158, 162]. This discovery depended on the prior localization of the sites of normal immunoglobulin chain genes at chromosomal bands 14q32, 2p12, and 22q11, the three sites involved in all common and variant Burkitt translocations. The fusion of the *MYC* gene to one of the immunoglobulin chain genes results in loss of *MYC* regulation and an inability to stop affected cells from entering the mitotic cycle, accounting for the high proliferative rate of the tumor cells and the dramatic increase in tumor size over hours or days.

It was unclear what role, if any, Epstein–Barr virus infection plays in the development of the chromosome injury. One proposal was that the virus infection of B lymphocytes increases their proliferation and, thereby, the probability of the chromosome alteration. This proposal, however, is too simplistic to explain the invariable involvement of the *MYC* gene and immunoglobulin loci.

Shortly after the elucidation of the role of the *MYC* gene in Burkitt lymphoma, Yoshihide Tsujimoto and associates cloned the breakpoint of the 14;18 translocation characteristic of the cells of follicular lymphoma [163]. The breakpoints involved were at the junction of a joining segment of the immunoglobulin heavy chain gene at chromosome 14q32, and the *BCL*-2 proto-oncogene (at l8q21), the protein product of which inhibits programmed cell death [164]. Some years later, the 11;14 translocation characteristic of the cells of mantle cell lymphoma was found to juxtapose the *BCL-1* gene (the product of which is a cyclin that regulates the cell mitotic cycle) on chromosome 11q13 to the same immunoglobulin heavy chain gene (14q32) [165]. *BCL-6*, a proto-oncogene at chromosome 3q27, is translocated to several different chromosome sites in a proportion of large B-cell lymphomas [166]. In T-cell lymphomas, a frequent breakpoint is at chromosome 14ql1, the site at which the delta chain of the T-cell receptor is embedded in the T-cell receptor alpha chain gene [167]. Thus, stimulated by the findings of chromosome translocations involving proto-oncogenes in Burkitt lymphoma cells, various laboratories have uncovered prevalent translocations involving antigen receptor genes, immunoglobulin genes (in the case of B cells), and T-cell receptor genes (in the cases of T cells), as major factors in the cause of human lymphoma.

The stimulus to medical research provoked by this unusual African tumor was singular. Virology, immunology, molecular biology, cytogenetics, and cytokinetics all have benefited from the study of this highly proliferative neoplasm. Cancer therapy has also benefited. The disease could not be treated by surgical excision. A tumor that could double in size in a day or two was a therapeutic challenge, even when apparently localized. Furthermore, Uganda had no radiation therapy facility, then. Burkitt, however, could and did use antiproliferative drugs at hand, such as methotrexate, nitrogen mustard, and cyclophosphamide [168–170]. Joseph H. Burchenal (1912–2006), an early cancer therapist and chief of Sloan-Kettering's division of clinical chemotherapy in the 1960s, was in Nairobi with a team from Sloan-Kettering Cancer Institute and gave Burkitt a supply of methotrexate with instructions for its use in a multiple dose course based on the positive experience they had with its use in the treatment of choriocarcinoma [169]. Since patients who made the trek to his clinic in Kampala did not stay for more than a few days—if they survived, and because the untreated disease was rapidly progressive, Burkitt administered the drugs in short, intensive treatments after which the children disappeared with their families, presumably to die at home. He later recalled his astonishment at meeting some of these children on his epidemiological trips, active and free of

evidence of their tumors. The rapid proliferative rate of the neoplasm made it sometimes responsive to short courses of intensive chemotherapy and in a small number of early cases a prolonged remission resulted. Together with the success of high-dose methotrexate in choriocarcinoma, Burkitt's observations provided an impetus to the exploration of short-term, high-dose chemotherapy in other malignancies. In cases of acute leukemia, high-grade lymphoma, and testicular cancer, this approach has been especially helpful. In 1972, Burchenal and Burkitt, along with several others, received the Albert and Mary Lasker Foundation Award for clinical medical research for their work in cancer pharmacotherapy. Mary Woodard Lasker (1900–1994) was an influential American philanthropist and health activist. She worked to raise funds for medical research and founded the Albert and Mary Lasker Foundation, which gives recognition and monetary prizes to outstanding basic scientists and clinical medical investigators for a notable achievement.

Few people have had the impact that Burkitt had in oncology and few achieved it with so little public support. Working in a poor Third World country with limited medical facilities was not a likely setting for such important discoveries in cancer research. But Burkitt did not let this circumstance dampen his interest when confronted with an unknown and uncharacterized disease. With the limited resources at hand, largely his curiosity, insight, and determination, Burkitt lymphoma became well known throughout the world. Burkitt said to a medical historian in an interview, "I didn't go out to Africa for science but because of what I believed to be God's call, and I've no complaints. I gave a spoonful and got back a shovel full." He received many awards and honors, but never lost his perspective. He was quoted as saying "… attitudes are more important than abilities, motives… than methods, character … than cleverness…, perseverance … than power." In 1994 Trinity College, Dublin, established the Denis Burkitt Research Institute, posthumously.

Adult T-Cell Leukemia–Lymphoma and the Human T-Lymphocytotropic Virus-1

At the beginning of the twentieth century (circa 1908), Vilhelm Ellermann (1871–1924) and Oluf Bang (1881–1937) at the University of Copenhagen first demonstrated that avian leukosis virus could be transmitted into chickens by a cell-free filtrate resulting in leukemia. In 1910, Peyton Rous (1879–1970) at the Rockefeller Institute in New York City extended Bang and Ellerman's experiments to show that a cell-free filtrate could transmit a solid tumor, a sarcoma (as well as leukemia) to chickens. Chickens have unusual characteristics in the behavior of endogenous retroviruses making them a good model for these experiments. Approximately 20 years later, Richard Edwin Shope (1901–1966) discovered the cottontail rabbit papillomavirus, dubbed the Shope papillomavirus, the first mammalian tumor virus. These observations were disputed and neglected for several decades until in 1951 Ludwik Gross (1904–1999), working at the Bronx Veterans Administration Medical Center in New York City, isolated murine polyomavirus, which could cause a variety of salivary gland and other tumors in specific strains of newborn mice. This observation could not be replicated and was not accepted until the findings were confirmed by scientists at the National Institutes of Health by reproducing the experiments under precisely the same conditions as used by Gross. Subsequently, in rapid order Charlotte Friend (1921–1987), then at the Sloan-Kettering Institute for Cancer Research, Arnold Graffi (1910–2006), at the Paul Ehrlich Institute in Frankfort, John Bromley Moloney (b.1924), Frank J. Rauscher, Jr. (1932–1993), and Werner H. Kirsten (1925–1992), the latter three at the National Cancer Institute in the United States, and others established that murine malignancies could be transferred by what we now know to be tumor viruses. Rauscher was named Director of the National Cancer Institute in 1972 to help lead President Nixon's war on cancer. By 1961, electron microscopy of putative tumor virus particles uncovered ultrastructural features in animal tumors that permitted their separation into types A, B, and C; the latter type viral particles are found in the cells of leukemias, lymphomas, and some sarcomas in vertebrates.

Based on these discoveries, the National Cancer Institute in the United States established the Special Virus Leukemia Program, which evolved into the Virus Cancer Program, and received generous and enthusiastic support for nearly 30 years. Initially, it was thought that human malignant cells could be propagated readily in animals or in cell culture and then used to search for tumor-associated viruses. A large industry of animal and cell culture support services was funded and mice, dogs, and apes received inoculations of human leukemic tissue. Soon these rudimentary experiments were replaced by scientifically more advanced investigations, but, through it all, human cancer viruses remained elusive.

Great advances occurred during this era in understanding viral replication and virus infectivity. Reverse transcriptase was discovered independently by Howard Martin Temin (1934–1994) at the University of Wisconsin in Madison and David L. Baltimore (b.1938), working at the Massachusetts Institute of Technology in Boston, the discovery for which they shared the Nobel Prize for Physiology or Medicine in 1970 with Renato Dulbecco (1914–2012), Temin's mentor. Dulbecco discovered that the infection of normal cells with certain oncoviruses led to the incorporation of virus-derived genes into the host-cell genome, and that this event lead to the transformation of the cells (the acquisition of a tumor phenotype), setting the stage for Baltimore and Temin's

discovery. This enzyme, a requirement for the survival of RNA viruses in host cells, enables the formation of DNA complementary to the viral RNA, permitting the virus to leave a permanent residue within the infected cell's genome. This property was required for the malignant transformation of cells by oncogenic RNA viruses, later referred to as oncornaviruses. All of the avian and mammalian leukemia viruses fell into the category of RNA viruses and, thus, were found to contain a reverse transcriptase. Although oncornaviruses caused malignancies in mice, cats, chickens, cattle, and apes, by the mid-1970s they had not been found to cause a single human cancer. During almost 20 years, and despite massive expenditures, the goal of the Cancer Virus Program remained was not achieved. Enthusiasm was replaced by skepticism and ridicule, not the least of which came from classic virologists who had felt that the expensive program was ill-advised from its beginning.

In the 1970s, one laboratory in particular remained dedicated to the quest of finding a human leukemogenic virus [171]. Despite the increasing disenchantment of others in the virological community, and despite having been set back by reporting a contaminating virus to be the long-sought human tumor virus (their finding that an oncornavirus caused acute myelogenous leukemia was not confirmed), Robert Gallo (b.1937) and his associates sought to prove that RNA viruses could cause human leukemia. Gallo's laboratory found a cell-derived chemical that supported T-lymphocyte growth in culture [172]. This T-cell growth factor was identified (later further characterized and renamed interleukin-2) and, in combination with the T-cell mitogen, phytohemagglutinin, permitted the continuous growth of normal T lymphocytes in culture. It was then noted that some T lymphocytes could be cultured with interleukin-2 alone; they did not need phytohemagglutinin for immortalization. With this critical new growth factor, normal and malignant T cells could be sustained in long-term culture. In this setting, the lymph node and blood T lymphocytes of a patient, with what was thought to be cutaneous T-cell lymphoma at the time, were used to develop two cell lines. Both the lymph node and the blood cell lines were found to contain an oncornavirus in which the reverse transcriptase was unlike that of any other known virus. The report by Bernard J. Poiesz (b.1948), Robert Gallo, and colleagues in 1980 was the first conclusive isolation of a human tumor virus. This virus was called human T-lymphocytotropic virus, number one, commonly referred to as HTLV-1 [173, 174].

The discovery of the HTLV-1 retrovirus in the cells from a patient with a cutaneous T-cell lymphoma turned out to be remarkable since no more than one in one hundred such patients have serological evidence of exposure to this agent. In retrospect, it is probable that the patient had adult T-cell leukemia–lymphoma with prominent cutaneous involvement, which can simulate cutaneous T-cell lymphoma. At that time, physicians, especially in nonendemic areas, were not sufficiently aware of the features of adult T-cell leukemia–lymphoma and grouped all T-lymphocyte malignancies involving the skin in the category of cutaneous T-cell lymphoma.

In 1977, Takashi Uchiyama (b.1946), Junji Yodoi (b. 1946), Kiyoshi Takatsuki (b.1930), and colleagues, at the Kyoto University School of Medicine, described a leukemia that they called "adult T-cell leukemia," in which most of the patients were from the Southwestern islands of Japan, especially Kyushi [175, 176]. Although rare in the United States and Europe, the disease also was found to be endemic in the Caribbean basin and Central Africa. One of the characteristics of this newly discovered lymphocytic leukemia was the lobulated appearance of the neoplastic lymphocyte nuclei. In 1980, Isao Miyoshi established cell lines of the neoplastic lymphocytes by co-culturing them with cord blood lymphocytes. Later, it was discovered that the cell lines were largely from cord blood cells that had become infected with what was found to be the etiological retrovirus. In 1981, an antigen was detected in a cell line from a patient with the disease, which was not shared with any other lymphoid lines studied [177]. Antibodies to this antigen were present in all patients with adult T-cell leukemia and some healthy subjects in endemic areas but were not found in patients with other lymphoproliferative diseases and in only occasional healthy persons in nonendemic areas. A cell line from a patient with the disease contained type C viral particles; the putative unique virus was called the adult T-cell leukemia virus (ATLV). The provirus DNA was cloned from the cell line of the patient with human adult T-cell leukemia [178]. In 1982, Motoharu Seiki (b.1949), Mistuaki Yoshida (b.1939), and colleagues at the Department of Viral Oncology of the Cancer Institute in Tokyo reported the isolation and characterization of the virus that is the cause of this form of adult T-cell leukemia–lymphoma [179]. The virus was found to be identical to HTLV-1, described in Gallo's laboratory 2 years earlier. The virus was shown to be the cause of adult T-cell leukemia–lymphoma [180]. The American and Japanese groups had met at Lake Miwa, near Kyoto, in March 1981 and shared data and established a collegial working relationship. Later, they agreed to call the virus "HTLV-1" recognizing the Gallo laboratory's priority, and to call the disease, adult T-cell leukemia–lymphoma, so carefully described by the Japanese [174, 176]. The Japanese found that the virus was transmitted principally from mother to child (T cells in breast milk), spouse to spouse (T cells in semen), and by blood transfusion of infected lymphocytes. In Japan, pregnant women are tested for HTLV-1 antibodies and, if positive, bottle feeding of their infant is urged. The potential for the virus's transmission by blood transfusion has resulted in a Food and Drug Administration requirement that a test for HTLV-1 be included among the panel of microbial agents for

which screening of blood must be carried out. There has been no success preparing a human vaccine. William A. Blattner (b. 1943) at the United States National Cancer Institute and Guy Marie Gérard Blaudin de Thé (1930–2014) did epidemiological studies to indicate that the distribution of the disease in persons in the Caribbean region was dependent on the particular tribes in Africa to which the Caribbean residents were related.

Not only did the discovery of HTLV-1 resuscitate the field of human viral oncology, and silence the critics of the by then defunct Cancer Virus Program, but the retrovirological techniques and pathophysiological insights developed during the search for and discovery of HTLV-1 prepared the scientific world for research into the etiology, diagnosis, and management of the late twentieth century's greatest infectious disease challenge, the human immunodeficiency virus (HIV), another RNA (retrovirus) virus, and the cause of the acquired immunodeficiency syndrome (AIDS). The work in Gallo's laboratory resulted in a technology to grow primary cultures of human T lymphocytes with IL-2, the development of a sensitive and specific assay for reverse transcriptase as a surrogate marker for the presence of a retrovirus, the demonstration that humans could be and were infected with retroviruses, and that retroviruses could cause a human disease and, at least in this case, a human cancer, among other important results. Dispute surrounded the priority of discovery of the human immunodeficiency virus, the discovery of which has been assigned to the laboratory of Luc Antoine Montagnie (b. 1932), a French scientist. Robert Gallo's laboratory's identification of prototypic human T-lymphocytotropic virus facilitated the discovery of the human immunodeficiency virus. Together with his colleague Françoise Barré-Sinoussi (b.1947), director of the Unité de Régulation des Infections Rétrovirales at the Institut Pasteur in Paris, Montagnier was awarded the 2008 Nobel Prize in Physiology or Medicine for his discovery of the human immunodeficiency virus. Harald zur Hausen (b.1936), a German virologist, also shared the Prize for his discovery that the human papilloma virus leads to cervical cancer. Robert Gallo was not recognized by the Nobel Foundation.

Helicobacter pylori-Related Lymphoma

At the beginning of the twentieth century, human cancer was well recognized but was a disease of mysterious origins. At a time of explosion of progress in identifying the causes of infectious diseases, medicine had no framework within which to place these sudden and unwelcome intrusions on human health. It was thought that an irritant or repetitive injury to tissue might induce uncontrolled growth of cells. Factors such as extreme heat, chemical exposures, radiation, or severe recurrent friction might be involved.

In 1926, Johannes Andreas Grib Fibiger (1867–1928), a Danish physician and professor of pathological anatomy, received the Nobel Prize in Physiology or Medicine for experiments he began in 1907. He conducted what by today's standards was a bizarre set of experiments that he interpreted as proving that gastric carcinoma in rats was caused by the larvae of a worm he named *Spiroptera carcinoma.* He found that the larvae acted as a carcinogen if fed to rats as the parasites of an unusual species of cockroach! He argued that after ingestion of the cockroaches, the larvae matured in the intestines of the rat leading to stomach cancer. The Nobel Committee highlighted the importance of the development of animal models to study cancer and the need to learn more of the external, possibly preventable, causes of the dread diseases. Although this may be the only Nobel Prize in Physiology or Medicine for work that was not confirmed, perhaps it served some unintended positive purpose. It is a curious irony that a spiral bacterium was found to be a cause for stomach cancer 90 years later. A greater irony was that in 1915 Katsusaburo Yamagiwa (1863–1930) induced squamous cell carcinoma by painting crude coal tar on the inner surface of rabbits' ears. Yamagiwa's work became the prototype for research on exogenous carcinogens but was not recognized by the Nobel Foundation in favor of the cockroach's parasitic larvae.

By the post World War I period, coal tar, aniline dyes, chimney soot, X-radiation, and some other agents had been linked to the causation of human cancer. In the early twentieth century, Theodor Heinrich Boveri (1862–1915), a German biologist, had brilliant insights into the importance of quantitative and qualitative changes in chromosomes in the pathogenesis of malignant tumors. He focused his interest on embryonic development in the nucleus of the cell. By the late nineteenth century, mitosis and chromosome movement during mitosis had been described. Mendelian genetics was being revisited and the question of the units of heredity was being explored. Boveri discovered the centrosome and its role in mitosis. He reasoned that a cancerous tumor may begin within a single cell in which chromosomes become disordered, causing the cells to divide uncontrollably [181]. He developed this remarkably prescient idea of the development of cancer from his study of the development of sea urchin eggs, manipulated experimentally, such that he could determine the normalcy of embryonic development from blastomeres with normal or abnormal chromosome complements. The origin of neoplasms in the somatic mutations of a single cell derived from the discovery of random inactivation of either the maternally or paternally derived X chromosome in the mammalian female, early in embryogenesis, and the use of that event to determine the unicellular origin of neoplasms in informative women who were heterozygous for a protein encoding X-linked gene. Inactivation of the X chromosome was first established by Mary Lyon (1925–2014),

a British geneticist, studying the genetic basis of patchy mouse coat color and, soon thereafter, in the human female using the X-linked enzyme glucose-phosphate dehydrogenase by Ernest Beutler (1928–2008), an American physician-scientist. The latter X-linked enzyme was used, initially, to demonstrate the single cell origin of neoplasms by David Linder (1923–1999), a pathologist and postdoctoral fellow, and Stanley Michael Gartler (b.1923), a geneticist at the University of Washington in 1965. The history and significance of the discovery of the X chromosome, its random inactivation in females, and the application of the latter phenomenon to identifying monoclonal tissue growths (neoplasms) are reviewed in [182].

In contrast to Fibiger's recognition by the Nobel Committee for an infectious etiology of cancer, the report by Peyton Rous (1879–1970) in 1911, working at the Rockefeller Institute for Medical Research that cell-free filtrates from chicken sarcomas could induce sarcomas in unaffected chickens, was met with marked skepticism by his peers and Rous changed his research direction [183]. After tumor viruses were established beyond question, including the Rous sarcoma virus, and the study of DNA and RNA tumor viruses became a centerpiece of cancer research, Rous was belatedly awarded the Nobel Prize in Physiology or Medicine in 1966, at age 85, 56 years after his discovery. Happily, his longevity permitted correction of the Nobel Committee's oversight, since the Nobel Prize is never awarded posthumously.

Today, human viruses are established as "causal" agents in human cancer in the epidemiological sense. The definition being that in their absence the disease would not occur. Papilloma virus, Epstein–Barr virus, hepatitis B and C viruses, human immunodeficiency virus, and HTLV-1 have been established as either causative or predisposing agents or are of high probability of being found to be so.

In 1981, William Richard Shaboe Doll (1912–2005) and his colleague Richard Peto (b.1943) published a treatise on the causes of cancer and concluded at that time that about 5% of cancers were the result of infections, principally viral, but that at least another 5% probably were induced by infectious agents [184]. Their estimates preceded the evidence for the role of several additional viruses in human cancer causation. They also suggested that bacteria in the intestines could convert nitrates to nitrites or to *N*-nitroso compounds that are potential carcinogens and that schistosomiasis in parts of Africa and clonorchiasis in China may be etiological in the development of bladder cancer and cholangiocarcinoma, respectively. Doll was among the foremost epidemiologist of the twentieth century being credited with establishing the link between smoking and lung cancer through a prospective study in British physicians in the 1950s. He also confirmed the link between smoking and heart disease.

In 1979, the possible direct relationship of bacteria to gastric cancer resurfaced, approximately 70 years after the start of Fibiger's improbable experiments. In 1983, J. Robin Warren (b. 1937) reported Campylobacter-like bacteria in the gastric antral mucosa in biopsies of patients with active gastritis [185]. Despite the prevailing view that the acidic gastric environment was hostile to bacteria with the exception of mycobacteria and other related "acid fast" organisms, careful analysis of the mucosa using silver stains demonstrated colonization of the surface epithelium in the gastric lining by small S-shaped bacteria. The bacteria were closely associated with antral gastritis. The mucosa was infiltrated with neutrophils and smaller concentrations of lymphocytes and plasma cells. Warren lamented the prior inattention to the microbiology of the stomach, and although the organisms looked like Campylobacter, he felt they were unclassifiable. Simultaneously, Barry J. Marshall (b. 1951), Warren's colleague at The Royal Perth Hospital, using techniques to grow Campylobacter, was able to culture the organisms and characterize their ultrastructure. He alluded to their potential significance as a cause of inflammatory and neoplastic disease in the stomach [186]. The two scientists speculated that a close and, perhaps, causal relationship existed between the organisms and either gastritis, gastric ulcer, or gastric cancer.

At least six reports of "spirochetes" or bacteria observed under the light microscope and associated with stomach ulcers or inflammation had been published from 1874 to the time that Warren and Marshall unequivocally found and grew their strange microaerophilic, multiflagellated, argyrophilic bacteria. Perhaps, the two most notable were, first, the report of Abraham Stone Freedberg (1908–2009) and a colleague in 1940 describing spirochetes in the gastric mucosa associated with gastric ulcers [187]. Freedberg had identified spiral organisms in about one-third of sections of stomachs removed for ulcers he received from surgery. Attempts to culture the organisms failed. His work was discouraged by his mentors because of the acceptance that ulcers were the result of stress. (Medical science has been more often the victim of hypotheses regarding stress as an etiology of disease than it has been the beneficiary.) In the discussion after Freedberg's paper, Frank DeVore Gorham (b.1888) described the microbiological basis on which he used intramuscular bismuth for chronic gastric ulcers that refused to heal. Today, bismuth is one of the antimicrobial agents used for the purpose of treating *Helicobacter pylori*-related peptic ulcer. Freedberg went on to become chief of medicine and cardiology at the Beth Israel Hospital, an affiliate of the Harvard Medical School. About 60 years was lost in the search for the cause of peptic ulcers, gastric carcinoma, and gastric lymphoma, as a result of the skepticism and lack of encouragement by Freedberg's advisors. In 1975, Howard William Steer (b.1943) and Duncan G. Colin-Jones observed that

80% of 50 patients with gastric ulcers they examined had gram-negative bacteria in the gastric mucosa and the bacteria did not disappear after the ulcers healed with treatment, in keeping with later observations that histamine-2 receptor antagonists, such as cimetidine, resulted in healing of ulcer craters but not the antral gastritis and duodenitis and was associated with a high ulcer relapse rate [188]. Unbeknownst to the therapist, the underlying microbial incitant had not been eradicated.

The spiral gastric bacterium, referred to as *Campylobacter pyloris* (né *pylorides*), was shown to be incompatible structurally and biochemically with that genus. In 1989, in recognition of these dissimilarities a new genus, *Helicobacter*, was established and the organism renamed *Helicobacter pylori*. A series of papers from 1989 to 1992 provided strong circumstantial evidence that *Helicobacter pylori* is causally associated with about two-thirds of patients with carcinoma of the stomach, worldwide [189]. In 1988, Judith Irene Wyatt (b.1956) and Barrie John Rathbone (b.1955) [190] working in the Department of Pathology at St James University Hospital, Leeds, and in 1989 Manfred Stolte and Sebastian Eidt [191], then at the Institute of Pathology, University of Cologne, had shown that the development of organized lymphatic tissue in the gastric mucosa is nearly invariably a response to *Helicobacter pylori* infection. In 1992, Andrew Charles Wotherspoon (b. 1960) and colleagues, working in the Department of Histopathology at the Royal Marsden Hospital, London, reported that one-third of patients with *Helicobacter pylori*-associated gastritis had prominent lymphoid follicles in the gastric wall and that eight had pathological features of mucosa-associated lymphoid tissue lymphoma [192]. The normal stomach does not harbor any lymphatic tissue.

Primary lymphoma of the stomach closely simulates the features of mucosa-associated lymphoid tissue (MALT) lymphoma [193]. These lymphomas arise in the stomach, salivary gland, or thyroid and require a promoting factor to induce lymphoid infiltration, accumulation, and organization, since these sites are normally devoid of lymphoid tissue. Autoimmune reactions account for the evolution of lymphoid tissue in the salivary gland (Sjögren's syndrome) and the thyroid (Hashimoto's syndrome). In that setting, a B-lymphocytic mucosa-associated lymphoma may develop. In their classic paper, Wotherspoon and colleagues also observed that among 100 cases of gastric mucosa-associated lymphoma, 92% of patients had evidence of *Helicobacter pylori* infection. In striking validation of the potential role of bacterial antigen in provoking these lymphoid tumors, treatment of six patients with B-cell gastric lymphoma and *Helicobacter pylori* infection with a combination of antimicrobial drugs resulted in disappearance of the lymphoma in five subjects and near disappearance in a sixth [192].

Wotherspoon and coworker's implication that *Helicobacter pylori* is the "cause" of gastric B-cell lymphoma of the stomach is built on the work of several prior observations and their own discoveries: the identification of the organism in the stomach, the culture and characterization of the organism, the association of the organism with antral gastritis, the association of the organism with the development of gastric mucosal lymphoid tissue, which is otherwise absent, and the association of the organism with gastric carcinoma. Unlike Fibiger's hypothesis regarding a microbial causation for gastric cancer in the rat, 85 years ago, the evidence supporting the Wotherspoon hypothesis is more compelling. This remarkable relationship provided the opportunity for both novel treatment and prevention of this lymphoma.

In 2005, Barry J. Marshall (b. 1951) and J. Robin Warren (b.1937) shared the Nobel Prize in Physiology or Medicine for discovering *Helicobacter pylori*, leading to the awareness of its significance in the cause of inflammatory and neoplastic diseases [194].

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38 Genetics in Lymphomagenesis

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Introduction

Lymphomas are a heterogeneous group of over 60 neoplasms that arise from lymphocytes and produce tumors in the lymph nodes, lymphatic organs and extranodal lymphatic tissue (i.e., lymphomas), as well as the bone marrow (i.e., multiple myeloma) or peripheral blood (i.e., leukemias). Lymphoid neoplasms as a group are some of the most common cancers in the US, with an estimated 112,380 cases in 2016 [1].

Lymphoma subtypes are considered to be clonal tumors of immature or mature B-cells, T-cells, or natural killer (NK) cells arrested at various stages of differentiation [2]. B-cell neoplasms appear to recapitulate the normal stages of B-cell differentiation, and many B-cell lymphomas can be linked to a presumed normal cell counterpart. The normal counterparts of T-cells and NK-cells are not as well characterized as B-cells, but they do share many immunophenotypic and functional properties and are currently grouped together. Based on this, the current World Health Organization (WHO) classification system [3], which has been incorporated into the International Classification of Diseases—Oncology (ICD-O) [4], recognizes precursor lymphoid neoplasm (B- and T-cell), mature B-cell neoplasms, mature T-cell and natural killer (NK)-cell neoplasms, Hodgkin lymphoma (HL), and histocytic and dendritic cell neoplasms. Acute and chronic lymphocytic leukemia (ALL and CLL, respectively), and multiple myeloma (MM) are classified as B-cell neoplasms [2]. A revision of the 4th edition of the WHO Classification was recently released, and although it did not allow for any new definitive entities, it incorporated new genetic/molecular and clinical data into current disease entities and added a limited number of new provisional entities [5].

From a tumor genomics perspective, our current understanding supports the hypothesis that lymphomas are aberrant outcomes of normal physiologic mechanisms used by both the innate and the adaptive immune system [6]. In the bone marrow and thymus, V(D)J gene recombination, which involves DNA double strand breaks, is used by both immature B- and T-cells for the assembly of immunoglobulin heavy- and light-chain (B-cells) and T-cell receptor α and β chain (T-cells) loci. Once B-cells have matured from the bone marrow, they migrate to peripheral lymphoid organs, where they can go through the germinal center reaction, which includes clonal expansion, somatic hypermutation affecting the variable regions of Ig genes, class-switch recombination, and affinity maturation/apoptosis. While these physiologic mechanisms allow for the creation of immense antibody diversity and specificity, they come with a trade-off of susceptibility to neoplasia mainly by generating reciprocal chromosomal translocations (leading to dysregulated expression of proto-oncogenes placed near Ig enhancers) and off-target somatic hypermutation of proto-oncogenes, ultimately leading to aberrant expression of oncogenes and/or inactivation of tumor suppressor genes [7, 8]. Thus, chromosomal translocations involving Ig genes and several partners are a hallmark of many lymphoma subtypes [6]. Additional drivers of lymphomagenesis include chromosomal copy number alterations (gains and deletions) and somatic mutations.

From a host perspective, while there has been a long history of case reports of familial clustering of lymphomas and leukemias, it has only been relatively recently that these malignancies were considered to have an important inherited genetic component outside of very rare hereditary cancer syndromes [9, 10]. Most of our current understanding of the genetic epidemiology of lymphoid neoplasms has been from

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studies of familial clustering and use of genome-wide association studies (GWAS), and most of this has been focused on understanding etiology, although there are growing data on host genetics in response to therapy and prognosis as well.

In this chapter, we first review the tumor (somatic) genomics of the major lymphoma subtypes, excluding MM, with a focus on somatic mutations identified through next-generation sequencing (NGS) and related technologies. We next review the strongest data addressing familial predisposition (including twin, case-control, and registry-based studies) to lymphomas broadly defined (i.e., HL, NHL, CLL/SLL, and MM). Finally, we review host (germline) genetics (including family and genetic association studies) for lymphoid neoplasms covering the major lymphoma subtypes (excluding MM). Within each section, we address both etiology (risk) and prognosis (outcome). Finally, we complete the chapter with a discussion of some clinical implications and future directions.

Tumor (Somatic) Genomics

NGS technologies have played an essential role in our understanding of the somatic genomic landscape of hematologic malignancies. Compared to Sanger sequencing, NGS provides numerous advantages, including the higher sensitivity and more comprehensive scope, allowing the detection and quantification of small subclones and characterizing the tumor architecture and evolution in an unprecedented fashion [11]. Many different approaches have been developed for DNA sequencing, from targeted sequencing to whole-exome sequencing (WES) and whole-genome sequencing (WGS). These comprehensive approaches allow the simultaneous detection of single nucleotide variants (SNV), small insertions and deletions (INDELs), as well as copy number alterations (CNAs). Furthermore, modification of WGS, such as mate-pair sequencing, has been successfully used in the detection of structural variants and complex genomic rearrangements. Similar advances have been observed in the study of the transcriptome through RNA sequencing (RNA-Seq), leading to the simultaneous screening of gene expression, alternative gene-spliced transcripts, gene fusions, and mutations/single-nucleotide polymorphisms (SNPs) [11]. Together, these technologies have significantly advanced our understanding of the molecular pathways that drive tumor development, helped to further classify tumor subtypes, and identified both prognostic and therapeutic genetic biomarkers. However, some technologic and analytic challenges remain, including management of large "omics" data sets, variation in the use of computational methods and analysis tools, and the public availability of data for comprehensive analysis [12]. The catalogue of somatic mutations in cancer (COSMIC) is the largest and most comprehensive resource for exploring mutations in human cancers. There are limitations to this resource however, as it only collects data that is described in original work and made publically available [13]. Nevertheless, because it is the broadest database for cancer mutations and expertly curated, we used information from COSMIC to generate a summary of the top 20 genes with mutations in each lymphoma subtype described below (Fig. 38.1). In this section, we further review the major somatic findings recently identified in lymphomas through NGS studies.

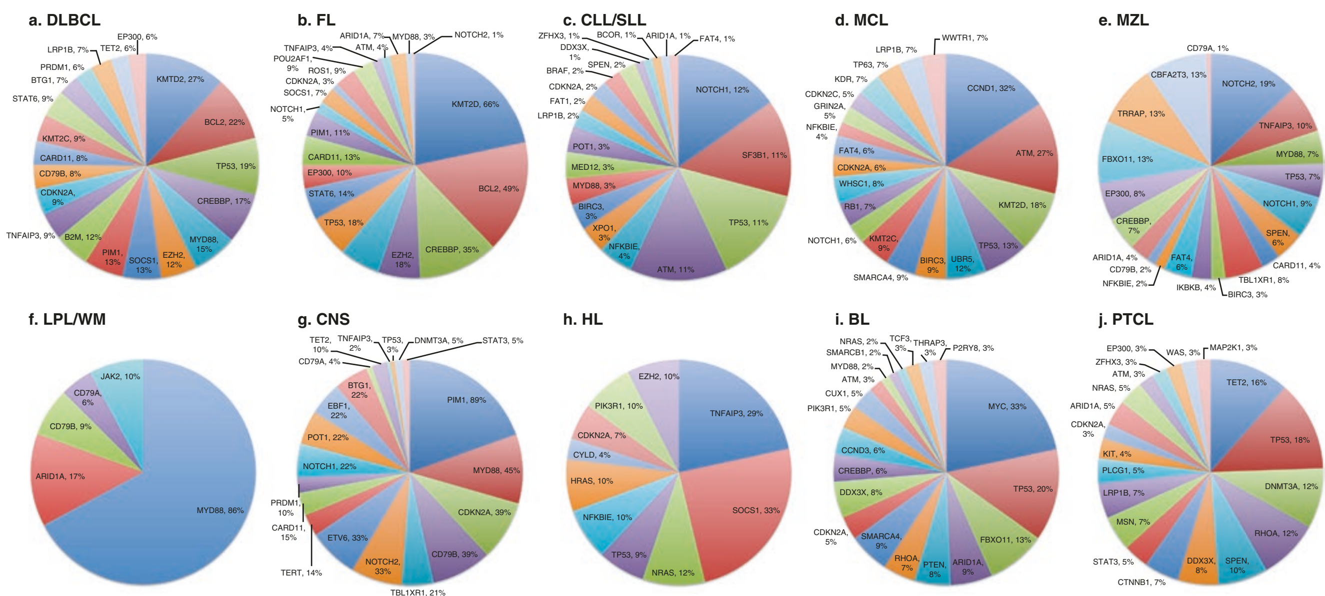

Fig. 38.1 The Mutational Landscape of Individual Hematologic Malignancies. Data on hematopoietic and lymphoid tissue, including lymphoid neoplasms, reported in COSMIC were used to graph the top 20 genes with mutations within each subtype

Diffuse Large B-Cell Lymphoma (DLBCL)

DLBCL is the most common NHL subtype, and is an aggressive lymphoma that can be potentially cured with immunochemotherapy in many patients. Advances in molecular profiling by gene expression profiling (GEP) allowed for the identification of major DLBCL subtypes defined by cell of origin (COO): germinal B-cell (GBC) subtype, an activated B-cell (ABC) type, and unclassifiable subtype [14]. Beyond COO, NGS of DLBCL tumors have revealed a heterogeneous genetic landscape with mutations in genes involved in antigen receptor/NF-κB signaling (*MYD88*, *CD79B*, *CARD11*, *TNFAIP3*, *PRDM1*), epigenetic regulation and chromatin modification, (*KMTD2*, *EZH2*, *CREBBP*, *EP300*, *MEF2B, HIST1H1C*), immune regulation (*B2M*, *CIITA*, *CD58*, CD70, *TNFRSF14*), cell cycle/apoptosis (*TP53, CDKN2A, FOXO1, GNA13, P2RY8, BCL2*, *MYC, BTG1*), and JAK/STAT signaling (*SOCS1, STAT3*) [15–19]. In addition to mutations, frequent CNAs include deletions at 17p (*TP53*), 9p21 (*CDKN2B*), 10q11 (*PTEN*), and 6q21 (*PRDM1, TNFAIP3*) and gains at 18q21 (*BCL2*) and 2p13 (*REL*) [20, 21]. The translocation t(14;18) has been detected in 12–30% of DLBCL patients [22–24].

Although a majority of the mutations identified in DLBCL are found in both COO subtypes, suggesting common mechanisms of pathogenesis, there are additional driver events unique to COO subtypes. ABC-DLBCL is frequently associated with constitutive activity of the NF-κB pathway and mutations in upstream activators such as *MYD88* [25], *CARD11* [26], and *CD79B* [27]. In contrast, GCB-DLBCL is dominated by mutations in *GNA13*, *P2RY8*, *TNFRSF14* and genes involved in epigenetic regulation and chromatin modification (*EZH2*, *CREBBP*, *EP300*, *CREBBP*). *MYC* rearrangements or *MYC* “double hits” (DH, *MYC* and *BCL2/IGH* fusion or a *BCL6* rearrangement) detected by FISH occur in 6–14% of DLBCL and are typically seen in GCB-DLBCL [28–31].

DLBCL heterogeneity is also exemplified by variability in treatment response. While a majority of DLBCL patients are potentially cured after front-line treatment with immunochemotherapy (R-CHOP), lack of remission or early relapse remains an important clinical issue [32]. WES data from 51 newly diagnosed and R-CHOP-treated DLBCL patients were used to explore the association of somatic genomic alterations with the novel endpoint event-free survival at 24 months (EFS24) [33] and a set of genes with mutations, chromosomal gains, and chromosomal losses were identified [34]. Integration of CNAs and mutation data revealed that 77% of patients who fail to achieve EFS24 have a combination of four variants: *FOXO1* mutation and gains in 3q27.3, 11q23.3, and 19q13.32. Because of the small sample size, further validation of these findings is needed.

Additional work has shown the significance of mutations in *TP53* [35, 36], *FOXO1* [37], and *MYD88* [38] with inferior DLBCL outcomes, and mutations in these genes were also more prevalent in cases that failed to achieve EFS24 [34]. Studies have shown that mutations in *TNFAIP3* and *GNA13* are linked to worse prognosis in R-CHOP-treated ABC [39]. Analyses have also been performed to identify genes that may be associated with disease relapse and *TP53*, *FOXO1, KMT2C, CCND3*, *NFKBIZ*, and *STAT6* emerged as top candidate genes implicated in therapeutic resistance [40]. Lastly, MYC-DH cases, which are associated with transcriptional dysregulation of c-myc, have been correlated with poor prognosis in patients treated with chemotherapy alone or with the addition of rituximab [28, 30, 31, 41, 42].

Follicular Lymphoma (FL)

FL is the second most common form of NHL and often follows an indolent disease course with slow progression [43, 44]. However, patients may develop resistant disease and transformation to a more aggressive subtype of lymphoma occurs at a rate of 2–3% per year [45–48]. FL arises from germinal center B-cells and the most common molecular defect is the translocation t(14;18)(q32;q21), which is found in 85–90% of the cases and results in the *IGH-BCL2* leading to the overexpression of the anti-apoptotic oncogene *BCL2* [49, 50]. However, the t(14;18) is also observed in healthy individuals and FL patients who are in long-term remission [44] suggesting the existence of additional genomic alternations that impact disease course.

Recently, WES and WGS of FL tumors and follow-up validation studies have discovered that point mutations in genes involved in epigenetic regulation and chromatin modification, including *MLL2*, *EZH2*, *CREBBP*, *EP300*, and *MEF2B*, dominate the FL genomic landscape [16, 51, 52]. Mutations in *JAK-STAT* pathway genes and B-cell receptor (*BCR*)/*NF-κB* signaling genes have also been identified, but with lower frequencies. WES and WGS studies to date have primarily focused on identification of small site mutations that are recurrent in FL tumorigenesis. However, additional studies have focused on defining genetic events associated with tumor clonal evolution and transformation. Longitudinal analysis of FL tumors identified early driver mutations in chromatin modifying genes (*CREBBP*, *EZH2,* and *MLL2*), whereas mutations in *EBF1* and regulators of NF-κB signaling (*MYD88* and *TNFAIP3*) and *TNFRSF14* were gained at transformation [53, 54]. In addition to mutations, inactivation of *TP53* and deregulated expression of *MYC* is commonly associated with a transformation of FL to DLBCL [55].

More recently, efforts have been put forth to identify genetic predictors of outcome in FL. Targeted deep sequencing

of 74 genes in immunochemotherapy-treated FL identified a panel of 7 genes (*EZH2, ARID1A, MEF2B, EP300, FOXO1, CREBBP,* and *CARD11*), that when combined with the Follicular International Prognostic Index (FLIPI) and ECOG performance status ("m7-FLIPI") showed better prognostic value than the FLIPI alone [56]. Further, the m7-FLIPI also predicted progression of disease within 24 months after therapy in both the German Low Grade Lymphoma Study Group and a British Columbia cohort [57]. While extremely promising, these models were based on a candidate gene approach, using known "drivers," which can miss other predictors that can be identified only by a comprehensive and agnostic approach. These models also do not evaluate other tumor genomic alterations, most prominently CNAs.

Chronic Lymphocytic Leukemia/Small Lymphocytic Lymphoma (CLL/SLL)

CLL and SLL are indolent B-cell malignancies, with CLL presenting in the bone marrow or peripheral blood and SLL presenting in the peripheral lymphatic tissue. Two seminal WGS/WES studies [58, 59] of large cohorts ($N = 452$ and 538, respectively) of CLL patients have been recently conducted that provide over 90% power to detect recurring driver genes with >3% mutation frequency. Together, these studies and prior sequencing studies [60–66] have identified or confirmed 58 recurring somatic driver or copy number alterations with 91% of CLL patients having at least one driver alteration. The most commonly mutated genes (>5% CLL cases) included *NOTCH1, ATM, SF3B1,* and *TP53*, whereas the most common copy number alterations included the well-known structural variants del(13q), del(11q), del(17p), and tri(12). Combining these drivers, the main pathways that are frequently altered include B-cell receptor signaling, cell cycle regulation, apoptosis, DNA damage response, NF-κB signaling, NOTCH1 signaling, RNA metabolism, and chromatin remodeling. When correlating these drivers with clinical characteristics, higher mutation load was seen in CLL cases who have unmutated status in the immunoglobulin heavy-chain variable region (*IGHV*), a strong CLL prognostic marker, supporting the clinical aggressiveness of *IGHV* unmutated CLL. A number of these somatic mutations have been found to be associated with OS or time to first treatment independent of stage and IGHV mutational status [58, 59]. However, other than *TP53* mutational status or del(17p), none of the common drivers show independent prognostic information beyond a model that included clinical stage, age, *IGHV* mutational status, and serum β_2-microglobulin [67]. Studies are needed to determine whether any of these drivers are associated with response to therapy.

Mantle Cell Lymphoma (MCL)

MCL is a B-cell lymphoma that arises from naïve pre-germinal center cells originating from the mantle region of secondary lymphoid follicles. Two main morphologic subtypes have been identified, classical and blastoid, with poor prognosis observed in the blastoid variant. The cytogenetic hallmark of MCL is the translocation t(11;14)(q13;q32), which juxtaposes *CCND1* to the *IGH* locus resulting in overexpression of cyclin D1, a cell cycle regulator not normally expressed in B-cells [68, 69]. A rare subset of MCLs lacks a *CCND1* rearrangement and does not express cyclin D1. However, these cases have similar clinical behavior, biological features, gene expression profiles, and genomic complexity as that of cyclin D1-positive MCL [70, 71]. The cyclin D1-related genes *INK4A*, *CDK4*, and *RB1* are also genetically altered in MCL [72, 73] and contribute to dysregulation of the cell cycle. In addition to the role of *CCND1*, mutations or deletions in genes that regulate the DNA-damage response, including *ATM, TP53,* and *RB1*, are commonly found in MCL tumors [74–76]. Genetic alterations that impact the NF-κB pathway have also been identified and include deletion of *TNFAIP3*. Recent NGS studies have led to the discovery of additional oncogenic mechanisms. Mutations in *NOTCH1* [77], *UBR5* [78], as well as genes involved in chromatin modification (*KMTD2, KMTD3, MEF2B, WHSC1, SMARC4),* cell cycle/apoptosis *(POT1, SMC1A, BIRC3),* and NF-κB activation *(CARD1, TRAF21)* [77–81], have been identified. *SOX11*, which is expressed in virtually all aggressive MCL, has recently been recognized as a diagnostic and prognostic antigen in MCL and may play a central role in regulation of cell growth [82].

In addition to mutations, numerous structural and numerical chromosomal alterations are recurrent in MCL [83]. Most cases of the blastoid variant also have complex karyotypes with three or more chromosomal abnormalities in addition to t(11;14) [69, 84]. A high number of recurrent CNAs have been described in MCL, including gains at 3q, 8q, 15q, and 18q and loss at 1p, 6q, 8p, 9p, 9q 11q, 13q, and 17p [85, 86]. These regions encode for *ATM*, *TP53*, *MYC*, *BCL2*, and *CDKN2,* all of which are important for MCL pathogenesis. Gene expression profiling in MCL has revealed a proliferation gene signature that is associated with poor prognosis [87] and correlates with copy number loss at losses of 1p, 1q, 9p, and gain of 7p [85].

Marginal Zone B-Cell Lymphoma (MZL)

MZL comprises a heterogeneous group of indolent B-cell lymphomas with overlapping morphological and clinical characteristics, and can be divided into three subtypes: extranodal marginal zone lymphoma of MALT type

(MALT lymphoma), splenic marginal zone lymphoma (SMZL), and nodal marginal zone lymphoma (NMZL).

MALT lymphomas are associated with chronic microbial infection and/or autoimmune disorders and are originated at diverse mucosal sites, including the stomach, lung, ocular adnexa, and thyroid [88]. MALT lymphomas are mainly driven by chromosomal translocations leading to the constitutive activation of the NF-κB signaling pathways, a common pathogenesis pathway in MALT lymphomas [88–103]. The t(11;18)(q21;q21) causes fusion of the genes *BIRC3* (API2) and *MALT1*, resulting in the expression of an API2–MALT1 fusion protein, while the t(1;14)(p22;q32) and t(14;18)(q32;q21) result in the overexpression of *BCL10* and *MALT1*, respectively, as a result of juxtaposition with the *IGH* enhancer. The translocations are limited to MALT lymphomas, and are not seen in other MZL. Furthermore, *TNFAIP3* is recurrently inactivated by deletion and/or mutation in MALT lymphomas [96, 97, 104]. *TNFAIP3* alterations occurred mostly in ocular adnexa, salivary gland, and thyroid, but rarely in the lung, stomach, and skin [96, 104–106]. Additional genes associated with the NF-κB signaling pathway, including *CARD11, CD79B,* and *MYD88,* are mutated in a small subset of MALT lymphomas [25, 105, 107]. Additional driver genes corresponding to other cancer-relevant pathways, such as *TP53*, *KMT2D, NOTCH1,* and *NOTCH2,* are found mutated in 5–25% of cases [108, 109].

SMZL is an indolent malignancy of splenic B-lymphocytes characterized by splenomegaly, peripheral leukocytosis, and cytopenias. Mutations in *NOTCH2*, a gene required for marginal-zone B-cell development, represent the most frequent abnormality in SMZL, accounting for 20–25% of cases [110–113]. Most mutations are nonsense or frameshift indels in the C-terminal PEST domain. *NOTCH2* mutations are associated with adverse clinical outcome in SMZL [111]. *NOTCH2* mutations are rare or absent in the remaining late B-cell malignancies, reinforcing its specificity to SMZL pathogenesis. Furthermore, other members of the NOTCH pathway (*NOTCH1, SPEN,* and *DTX1*) are mutated in 5–10% of SMZL [113].

The Krüppel-like factor 2 gene (*KLF2*) is mutated in 20–40% of SMZL [110, 114], but rarely in related malignancies. KLF2 encodes a transcription factor essential for the homeostasis and differentiation of peripheral B-cell subsets [115, 116]. Most mutations are nonsense or frameshift indels, leading to inactivation of the capability of KLF2 to suppress NF-κB activation through TLR, BCR, BAFFR, and TNFR signaling [110]. The NF-κB signaling pathway is also affected by mutations in *IKBKB, TNFAIP3, BIRC3,* and *TRAF3,* which affect approximately a third of SMZL cases [113]. Collectively, mutations of genes regulating MZL development and NF-κB activation are found in 60–70% of SMZLs [111, 113]. Furthermore, genes regulating chromatin remodeling and transcriptional regulation are mutated in ~35% of SMZL [113]. The MLL2 histone methyltransferase (*KMT2D*) is mutated in 15%, *ARID1A*, member of the SWI-SNF chromatin remodeling family, in 10%, and the acetyltransferases *EP300* and *CREBBP*, in ~5% each [113]. SMZL is mainly characterized by the presence of recurrent deletions on 7q32-q35 [96, 104, 117–119]. Whole or partial gains of chromosomes 3 and 12 and losses of 17p have also been recurrently identified [119, 120].

NMZL is a rare and indolent malignancy that is distinguished from SMZL by the different pattern of dissemination. The knowledge surrounding the NMZL genomic landscape is very limited. The only comprehensive study performed in NMZL shows a large overlapping with SMZL, excepting the high prevalence of *PTPRD* mutations, otherwise absent in SMZL [121]. Mutations in *PTPRD*, a protein tyrosine phosphatase that regulates cell growth, cause loss of phosphatase activity, and are associated with increased proliferation index in NMZL. Furthermore, aberrant methylation of PTPRD-promoter CpG sites was found in cases with monoallelic mutation or deletion [121], suggesting epigenetic downregulation of the remaining allele. Studies in NMZL have identified the presence of whole or partial gains on chromosomes 3, 6p, 12, and 18 [95, 96], but no unique abnormalities have been identified in this subtype.

Lymphoplasmacytic Lymphoma/Waldenström Macroglobulinemia (LPL/WM)

LPL/WM is a rare neoplasm of small B lymphocytes, plasmacytoid lymphocytes, and plasma cells that commonly have an IgM paraproteinemia. One of the most representative paradigms of a cancer with a common somatic mutation shared by most patients is LPL/WM. *MYD88* shows the p.L265P mutation in nearly all LPL/WM patients [107]. Several studies using a variety of approaches confirmed the presence of *MYD88* p.L265P in 70–100% WM [122–129]. The same mutation is found in IgM monoclonal gammopathy of undetermined significance (IgM-MGUS), but only in ~50% of cases [125, 126]. Such difference in mutation prevalence could either support that p.L265P is a universal event required for the progression from IgM-MGUS to LPL/WM or the existence of different biologic subtypes of IgM-MGUS, with the p.L265P only found in cases evolving to WM [126, 130].

Allele specific PCR (AS-PCR) showed to be a useful tool for *MYD88* p.L265P screening in WM. Matched paired peripheral blood and bone marrow CD19+ populations showed comparable detection values of p.L265P by AS-PCR (89% in bone marrow versus 85% in peripheral blood) [131]. The results obtained from AS-PCR are promising, providing a less invasive method, eliminating the need for a bone marrow biopsy for molecular tests. Additionally, the mutation

screening has been indicated as a biomarker for measuring tumor burden and response assessment. Therefore, the identification p.L265P after therapy shows sensitivity comparable to flow cytometry [124].

Nonsense and frameshift mutations affecting C-X-C chemokine receptor type 4 (*CXCR4*) are found in nearly 30% of WM [129, 132, 133]. The mutations target the same hotspot previously described in warts, hypogammaglobulinemia, infections, and myelokathexis (WHIM). *CXCR4* mutations were associated with tumor growth and propagation to extramedullary organs [132]. An anti-CXCR4 antibody leads to tumor reduction in a C1013G/CXCR4 WM model, demonstrating its potential use as a therapeutic target [132]. Other recurrently mutated genes in WM included *ARID1A* (17%), *CD79B* (7–9%), *TP53* (7%), and *CD79A* (5%) [129, 134]. Further studies are needed to clarify the effect (if any) of such mutations in WM pathogenesis.

Regarding the importance of *MYD88* and *CXCR4* mutations in patient outcome, a recent study showed that LPL/WM patients with p.L265P presented at earlier age [133]. Furthermore, patients with mutation were associated with higher bone marrow involvement, higher serum IgM, and lower IgA and IgG levels [127]. Additionally, IgM-MGUS with p.L265P showed a higher risk of progression to WM [126]. Patients with mutations in both, *MYD88* and *CXCR4,* showed the highest BM involvement and serum IgM levels, compared with intermediate levels in cases with mutated *MYD88* and wild-type *CXCR4,* and low levels in patients without mutations in any of those genes [133]. Moreover, patients with both mutations presented more commonly with symptomatic disease requiring therapy at diagnosis [133]. On the other hand, *CXCR4* mutations were not associated with worse overall survival (OS).

MYD88 is a promising therapeutic target. In vitro studies show that *MYD88* p.L265P leads to the NF-κB signaling pathway activation through two independent pathways involving Bruton's tyrosine kinase (BTK) and IRAK1 and IRAK4 [135]. BTK is targeted by Ibrutinib, an FDA approved drug widely used in the treatment of CLL. In a recent clinical trial performed in WM patients who had received at least one previous therapy, the use of Ibrutinib was associated with a decreased IgM level and bone marrow involvement and an increased hemoglobin level [136]. The best response rates are found in patients with mutant *MYD88* and wild-type *CXCR4* (100% overall response rate and 91% major response rate), followed by double mutants (86% and 62%, respectively) and finally by patients without mutations in both genes (71% and 29%) [136]. Overall, *MYD88* p.L265P has the potential to become a powerful biomarker for diagnosis, prognosis, response assessment, and treatment in WM.

Regarding CNAs, LPL/WM has a very low-complexity karyotype, with a median of 3 chromosomal abnormalities per case [134, 137, 138]. Deletion of 6q is the most common chromosomal abnormality, reported in 30–50% of cases [134, 137–141]. Deletion of 6q has been associated with low albumin, high B2M [140, 142], anemia [142], and low IgM production [143, 144]. No association has been found between the deletion and response rate, progression-free survival (PFS) or OS. Trisomy 4, found in nearly 20% of LPL/WM, is a distinctive feature of LPL/WM compared with the remaining low-grade B-cell lymphomas [137, 138, 140, 145]. Additional recurrent abnormalities found between 5–20% of LPL/WM include gains of chromosomes 3, 6p, 12, and 18 and deletions of 7q, 11q23 (*ATM*), 13q14 (*MIRN15a-16*), 17p13 (*TP53*), and Xq26 [95, 104, 137, 138, 140, 141, 146, 147]. Translocations involving IgH locus are rare in LPL/WM, found in less than 3% of cases [140, 141, 143, 145].

Primary Central Nervous System Lymphomas (PCNSL)

PCNSLs are extranodal NHLs confined to the central nervous system, and the majority of cases (90%) are observed in immunocompetent individuals. PCNSLs show a considerable genetic heterogeneity, affecting multiple genes and pathways. Genomic data integration highlights that practically all patients show genetic lesions affecting a variety of signaling pathways that share their ability to induce the NF-κB transcription complex, including the B-cell receptor (BCR), CD40, and Toll-like receptor (TLR) pathways. The most commonly mutated gene is *MYD88*, found altered in up to 90% of cases [148–153]. The mutational hotspots are similar to nodal ABC-DLBCL, although the mutation prevalence is nearly three times more frequent in PCNSLs [16, 25, 52]. Several other genes of the BCR/TLR/NF-κB pathways are recurrently mutated, especially *CD79B*, which is found mutated in approximately 40–60% of PCNSL [148–152]. The high prevalence of concurrent *CD79B* and *MYD88* mutations is an additional distinguishing feature of PCNSLs compared with ABC-DLBCLs [148, 149]. Other recurrently mutated genes in the NF-κB signaling pathway are *CARD11, TNFAIP3,* and *TBL1XR1*, found in 20–30% of cases each [148–152].

Immune surveillance is another pathway recurrently affected in PCNSL. Therefore, Human Leukocyte Antigen (HLA) genes are found deleted in 50–80% of cases [149, 153, 154], *B2M* and *CD58* are mutated and/or deleted in 20–30% [148, 149, 154], and *CIITA* has been involved in chromosomal translocations [148]. More recently, copy gain and translocations involving *PD-L1* and *PD-L2* have been reported [148].

PRDM1, a crucial gene in lymphocyte differentiation is impaired in nearly 30% of PCNSL. Even though only small cohorts have been analyzed so far, independent survival

analyses suggested that loss of *PRDM1* is a poor prognosis marker in PCNSL [149, 153]. Another key gene in lymphocyte differentiation recurrently mutated is *IRF4*, which has been mutated in 10–20% of cases [148, 149, 152]. Several other genes associated with relevant pathways are also shared by nodal DLBCL, such as *GNA13, TMEM30A, BTG1,* and *BTG2*, between others. Furthermore, SHM also targets genes at high frequency in PCNSL, such as *PIM1* and *MYC* [149, 150, 152, 155]. Several recurrent chromosomal abnormalities have been identified in PCNSL, including translocations of *IgH-BCL6* [156], deletions of 6p21, 6q, 8q12, and 9p21, and gains of 7q, 11q, and chromosome 12 [149, 154, 157–159].

Despite all these similarities with ABC-DLBCL, there is a subset of PCSNL-specific alterations, thus reinforcing the idea that PCNSLs are a pathogenically distinct group. In contrast to nodal DLBCL, PCNSLs rarely have *TP53* mutations, although p53 is indirectly impacted via loss of *CDKN2A*, which has been described as mutated and/or biallelically deleted in more than half of PCNSL cases [148, 149, 153, 154]. Other features unique to PCNSL include recurrent abnormalities in *PRKCD* and *TOX* [149, 152–154]. *PRKCD*, a proapoptotic protein kinase, is associated with a wide variety of cellular processes, including growth, differentiation, secretion, apoptosis, and tumor development [160–163]. Moreover, several studies have suggested that loss of *PRKCD* is associated with defective B-cell apoptosis, B-cell hyperproliferation, and autoimmune disorders [164–167]. Another interesting finding is the identification of recurrent alterations affecting *TOX* [149, 153, 154], a gene that plays a key role in T-cell development [168, 169]. The absence of *TOX* prevents CD4 lineage T-cell development, including natural killer T (NKT) and T regulatory (Treg) CD4 T-cell sublineages, but has only modest effects on CD8 T-cell development [170]. Furthermore, TOX-deficient mice (TKO) lacked lymph nodes and had a significant decrease in the frequency and size of Peyer's patches [171]. A recent study provides data that support an important role of *TOX* expression in B-cell biology [172].

Hodgkin Lymphoma (HL)

Classical HL (cHL), which compromises approximately 95% of all HL [3], involves various inflammatory cells with only about 1% of the cellular infiltrate being the neoplastic Hodgkin and Reed-Sternberg (HRS) tumor cells [173]. The low prevalence of HRS cells brought into question the cellular origin of this disease, however, molecular cloning techniques allowed for the discovery of its B-cell origin [174, 175]. HRS cells show dysregulation of numerous signaling pathways, which is partly mediated by cellular interactions in the lymphoma microenvironment and partly by genetic alterations. Epstein–Barr virus (EBV) has also been shown to play a pathogenic role in HL, although not all HRS cells are EBV positive [176].

Genetic analysis on cHL has been hampered by the low content of the HRS cell within the tumor microenvironment. However, cytogenetic analyses have identified recurrent chromosomal breakpoints on chromosome arms 1p, 6q, 7q, 11q, and 14q, recurrent gains of chromosomes 2, 5, 9, and 12 and losses on chromosomes 13, 21, and Y [177–183]. Using array-based comparative genomic hybridization (aCGH), HRS cell have recurrent gains on 2p12-16, 5q15-23, 6p22, 8q13, 8q24, 9p21-24, 9q34, 12q13-14, 17q12, 19p13, 19q13, and 20q11 whereas losses involved Xp21, 6q23-24, and 13q22 [184].

Constitutive activation of the NF-κB signaling pathway is central to proliferation and survival of HRS cells [185]. This finding is supported by genetic studies identifying mutations in the NF-κB negative regulators *NFKBIA* and *NFKBIE* in 10–20% of cases [186, 187]. Genomic gains of *REL* and *BCL11A* are present in about 30% of HL [188, 189] and chromosomal loss or inactivating mutations in *TNFAIP3* are also commonly found [190, 191]. Genetic alterations in other regulators of NF-κB, including *TRAF3*, *BCL3*, and *CYLD,* are also present in HRS, but with less frequency [180, 192, 193].

More recent data point to an important role for immune evasion in the survival of HRS cells. The genes encoding the PD-1 ligands, *PDL1* and *PDL2* (also called *CD274* and *PDCD1LG2,* respectively), are targets of chromosome 9p24.1 amplification, a recurrent genetic abnormality in HRS cells [194]. The 9p24.1 amplicon also includes *JAK2.* Chromosomal amplification leads to overexpression of the PD-1 ligands on HRS cells and supporting data suggests that EVB infection also increases the expression of PD-1 ligands in EBV-positive HL [195]. These studies have led to the use of PD-1 blockade (Nivolumab) as a highly effective therapy in relapsed or refractory HL [196].

Burkitt Lymphoma (BL)

BL is derived from germinal center B-cells [197] and is classified into three distinct entities based on clinical presentation, morphology, and biology. Endemic BL (eBL) represents the most common childhood malignancy in equatorial Africa and is associated with EBV, nearly all tumor cells carry the viral genome. Sporadic BL (sBL), which is histologically indistinguishable from eBL, is seen throughout the world with only 10–20% of tumors being EBV positive. A third BL variant, immunodeficiency related-BL, was identified in patients with HIV/AIDS [3, 198].

A hallmark feature of BL is the presence of the translocation t(8;14) [199], juxtaposing *IGH* and *MYC* [200, 201].

MYC translocation partners also include *IGK* at 2p11 and *IGL* at 22q11 [202]. The identification of *MYC* deregulation in BL and the characterization of *MYC* transgenic mice clearly demonstrated a central role for MYC overexpression in lymphoma pathogenesis [203]. However, the delay in lymphoma development in the *MYC* transgenic mouse model suggests that *MYC* alone is not sufficient for malignant transformation. Inactivating mutations in *TP53* are common in BL and occur in 35% of cases [204, 205]. Next-generation sequencing of BL has revealed novel oncogenic mechanisms and identified mutations in *CCND3*, *CDKN2A*, *IDH3,* and *TCF3* [206–208].

Peripheral T-Cell Lymphomas (PTCL)

PTCL are composed by a heterogeneous group of generally aggressive NHL originated from post-thymic lymphocytes. Adult T-cell leukemia/lymphoma (ATL) is a PTCL associated with human T-cell leukemia virus type-1 infection. Almost all ATL are characterized by genetic abnormalities affecting the TCR signaling, the NF-κB signaling or downstream pathways [209]. *PLCG1*, which encodes the phospholipase PLCγ1, a key regulator of proximal TCR signaling, is mutated in 35% of ATLs [209]. Other recurrent mutations affecting the TCR pathway include *VAV1* (18%) and *FYN* (4%). *VAV1* encodes a guanine nucleotide exchange factor that is phosphorylated by the Src family kinases (including FYN) and interacts with PLCγ1 [209]. *PRKCB*, which encodes PKCβ, a signaling molecule downstream of PLCγ, is mutated in 33% of the cases. Downstream of PKC is located *CARD11*, which is mutated in 25% of ATL. Mutations in *CARD11* and *PRKCB* are significantly correlated, suggesting a synergistic effect between them [209]. Additional genes of the TCR–NF-κB signaling, including *CBLB, TRAF3, TNFAIP3, ERC1, IKBKB, RELA, NFKBIA,* and *PTPRC,* are also recurrently affected in ATL [209].

Another pathway connected with TCR signaling and recurrently affected in ATL is the B7-CD28 co-stimulatory signaling. Recurrent chimeric fusions affecting CD28 (CTLA4-CD28 and ICOS-CD28) were found by mRNAseq analyses [209]. B7-CD28 signaling molecules, including CD28, CTLA4, and ICOS co-receptors, are involved in TCR signaling regulation [210, 211].

Similar to B-cell malignancies [17, 148, 149, 154, 212], half of ATLs have genetic abnormalities affecting immune checkpoint genes, including *HLA* genes, *B2M, CD58, CD274, PDCD1,* and *FAS* [209]. Furthermore, genes involved in epigenetic regulation (*TET2, DNMT3A, IDH2, EP300, SETD2,* and *KDM6A*) are also recurrently affected in ATL. Another class of genes commonly affected in ATL is G protein-coupled receptors (GPCRs) involved in T-cell trafficking, including *CCR4*, *CCR7,* and *GPR183*, found mutated in 10–30% of ATL [209]. Additional altered pathways are the JAK-STAT (*JAK3, STAT3,* and *PTPN1*), NOTCH (*NOTCH1, ATXN1,* and *ZFP36L2*), PI3K-AKT (*PI3KCD* and *INPP4B*), and casein kinase 2 (*CSNK2A1* and *CSNK2B*) signaling pathway [209].

Many recurrent CNAs have been described in ATL, including gains of 2q33 (*CD28*), 6p25 (*IRF4*), 7q22 (*CARD11*), 10p15 (*PRKCQ*), 14q32 (*BCL11B*), and 19p13 (*SMARCA4*) and losses of 1p13 (*CD58*), 2q34 (*IKZF2*), 6p22 (*ATXN1*), 6q (*TNFAIP3*), 9p21 (*CDKN2A*), 10p14 (*GATA3*), and 12q12 (*ARID2*) [209].

Systemic anaplastic large cell lymphoma (ALCL) is an aggressive CD30$^+$ NHL. Approximately 60% of ALCLs show expression of protein ALK (ALK+). The genetics landscape associated with ALK+ ALCL is driven by translocations of the ALK proto-oncogene leading to ALK fusion proteins [213]. The most common translocation is the t(2;5) *NPM-ALK* found in 80% of cases [213]. The ALK chimeras activate STAT3, which is required for the maintenance of the neoplastic phenotype in ALK+ ALCL [214].

Interestingly, the initial genomics analysis on ALK-negative (ALK−) ALCL cases also identified recurrent genetic abnormalities leading to the activation of the JAK/STAT3 pathway, suggesting that the STAT3 activation is a unifying event in ALCL, regarding the ALK status. Therefore, activating mutations in the JAK-STAT pathway genes (*STAT3* and *JAK1*) are found in nearly 40% of the systemic ALK− ALCL cases [215]. Furthermore, mRNAseq screening of ALK− ALCL identified recurrent fusion transcripts of tyrosine kinases with different partners, including *NFKB2-ROAS1* and *NFKB2-TYK2* [215]. Additional studies identified the t(6;7) *DUSP22-IRF4* [216] and translocations involving *TP63* [217], in 30% and 8% of ALK− ALCL, respectively, but absent in ALK+ ALCL. Overall, ALK+ ALCL affects younger patients and has a more favorable clinical course than ALK-negative (ALK−) ALCL [218].

Angioimmunoblastic T-cell lymphoma (AITL) is a common subtype of PTCL with a poor prognosis. DNA sequencing analysis indicates a key role of epigenetic regulation in the pathogenesis of AITL, with almost 70% of cases showing mutations in *TET2, DNMT3A, RHOA,* and *IDH2* [219]. *IDH2* mutations are specific to AITL, whereas mutations of the other 3 genes are broadly distributed across other types of PTCL [220]. Furthermore, *FYN* (SRC family kinase), *ATM* (DNA damage response), *B2M, CD58* (immune surveillance), and *CD28* (TCR signaling regulation) are recurrently mutated in AITL [220, 221].

Sézary syndrome and Mycosis fungoides are cutaneous T-cell malignancies derived from CD4+ skin-homing T-cells [222]. Sézary syndrome shows mutations in genes involved in epigenetic regulation (*TET2*, *CREBBP*, *KMT2D*, *KMT2C*, *BRD9*, *SMARCA4,* and *CHD3*), NF-κB (*TNFR2, CARD11,* and *PRKG1*), and MAPK (*MAPK1* and *BRAF*) [223, 224].

Sezary is characterized by recurrent gains of chromosome 7, 8q, and 17q and losses of 2p23 (*DNMT3A*), 10q23 (*PTEN*), 12p13 (*CDKN1B*), 13q14 (*RB1*), and 17p13 (*TP53*) [223]. Analysis of mycosis fungoides identified somatic mutations in *SMARCA4, KMT2D,* and *KMT2C* [223].

Summary

Significant advances have been made in our understanding of the somatic genomic landscape of hematologic malignancies and this contributes to our understanding of disease biology, identifies unique subsets of tumors, provides insight on patient outcome, and identifies new therapeutic targets. Additionally, it allows us to comprehensively examine mutations across hematologic malignancies and identify common genes and pathways that are dysregulated. In Fig. 38.2, using data from COSMIC we show the top 20 genes most recurrently mutated across diseases. These data reveal that genes involved in NF-κB regulation (*MYD88*, *CD79B*, *TNFAIP3*, *CARD11*), DNA repair (*TP53*, *ATM*), control of the cell cycle (*CDKN2A*, *CCND1*, *PIM1*), histone modification (*KMT2D*, *EZH2*, *CREBBP*, *TET2*), gene transcription (*ETV6*, *ARID1A*), and the NOTCH pathway (*NOTCH1*, *NOTCH2*) are key drivers of hematologic malignancies.

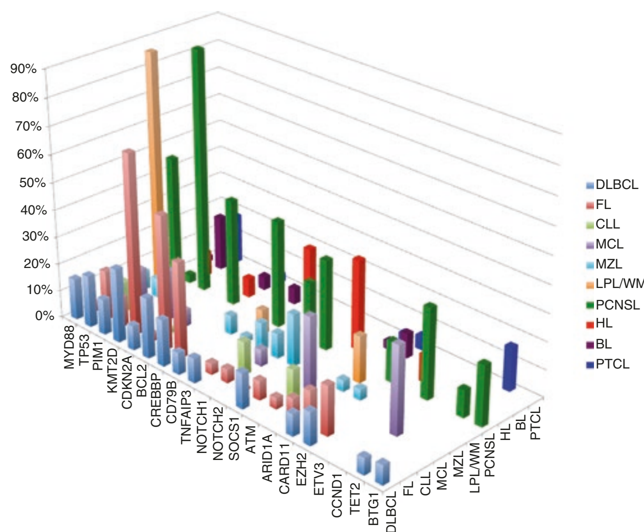

Fig. 38.2 Genes Mutated Across Hematologic Malignancies. Comparison of the most recurrently mutated genes across entities. These data highlight genes in NF-κB and NOTCH pathways, DNA repair, control of the cell cycle, histone modification and gene transcription

Familial Predisposition and Host Genetics

Environmental factors and somatic events are thought to be the predominant contributors to the development of lymphomas, and in uniformly treated patients, clinical characteristics along with the biology of the tumor clone (particularly genomic alterations) as well as the microenvironment are thought to be the predominant contributors to treatment response, lymphoma progression, and OS [225, 226]. However, there has been a growing interest in the role of inherited genetic susceptibility to lymphoma risk and prognosis. A variety of study designs have been used to identify inherited predisposition to lymphoma risk and prognosis, and new genotyping and sequencing technologies have allowed the identification of chromosomal locations underlying these associations.

Familial Predisposition

Etiology

Twin and Registry-Based Studies

In the largest published population-based study of over 200,000 twins from Denmark, Finland, Norway, and Sweden [227], familial cancer risk for non-acute leukemia (mainly CLL) was high in monozygotic (15.2%, 95% CI 6.1–33.2) versus dizygotic (4.1%, 95% CI 1.3–11.9) twins and heritability was estimated at 57% (95% CI 0–100%) with 0% estimated for shared environment. There were too few concordant twins with HL, NHL, or MM to estimate familial risk or heritability. In a twin study of lymphomas [228], there was a 100-fold higher risk of HL in monozygotic twins of patients with HL compared background rates (SIR = 99; 95% CI 48–182), while there was no excess risk in dizygotic twins; in contrast, there was a 23-fold higher risk of NHL in monozygotic twins of patients with NHL and a 14-fold higher risk in dizygotic twins, suggesting a stronger role for shared environment for NHL.

Based on data from the Utah Population Database and the Utah Cancer Registry [229], the risk of NHL was increased 1.7-fold in first-degree relatives of a proband with NHL (familial RR = 1.68, 95% CI 1.04–2.48); the risk of lymphocytic leukemia was >5-fold in first-degree relatives of a proband with lymphocytic leukemia (familial RR = 5.69, 95% CI 2.58–10.0); and the risk of MM was 4.3-fold higher in relatives of a proband with MM (familial RR = 4.34, 95% CI 1.97–7.63). In contrast, the risk of HL was only elevated 1.3-fold in first-degree relatives of a HL proband (familial RR = 1.27, 95% CI 0.12–3.65), although power for this estimate was low (only 2 exposed cases). Using updated data and a complementary analytic approach that estimates the Genealogical Index of Familiality [230], excess relatedness

was observed for NHL, CLL, and MM, but was only borderline significant for HL. CLL had the highest index of all cancer sites tested. For NHL, CLL, and MM, the excess relatedness was also observed for distant relatedness (i.e., for distant relatives), which may be interpreted as providing evidence that familial clustering is more likely due to shared genetic versus shared environmental contribution, as the latter would be lower in distant relationships.

The most comprehensive data available on familial aggregation by lymphoma subtypes has been published using registry data from Scandinavia (summarized across studies in Table 38.1). This approach compares the cancer experience in first-degree relatives of lymphoma patients with the cancer experience in relatives of matched population controls. First-degree relatives of cases with NHL were at higher risk of developing NHL (RR = 1.7) and HL (RR = 1.4), while this was slightly weaker for CLL (RR = 1.3) and not associated with MM (RR = 1.1) [231]. First-degree relatives of cases with CLL were at particularly high risk of CLL (RR = 8.5), as well as risk of NHL (RR = 1.9) and HL (RR = 1.5), but were not at increased risk of MM (RR = 1.2) [232]; first-degree relatives of cases with HL were at higher risk of developing HL (RR = 3.1) and CLL (RR = 2.1), while this was much weaker for NHL (RR = 1.3) and was not associated with MM (RR = 1.0) [233]. Consistent with these results, first-degree relatives of cases with MM were only at elevated risk of MM (RR = 2.1), with no excess risk of NHL, CLL, or HL [234].

The most recent analysis from five Nordic countries of 169,830 first-degree relatives of 45,406 NHL patients who were diagnosed from 1955 to 2010 reported that the lifetime cumulative risk of NHL was 1.6% in siblings (representing an SIR of 1.6, 95% CI 1.2–1.9) and 1.4% in parent–offspring pairs (representing an SIR of 1.4, 95% CI 1.3–1.5), and that familial risk did not significantly change by age at diagnosis of NHL in relatives [235]. In a similar analysis of 57,475 first-degree relatives of 13,922 HL patients, the lifetime cumulative risk of HL was 0.9% (representing an SIR of 6.0, 95% CI 4.8–7.4) and 0.4% in parent–offspring pairs (representing an SIR of 2.1, 95% CI 1.6–2.6), and familial risk was higher for sisters (versus brothers or unlike-sex siblings) and for younger age at diagnosis of HL in relatives [236].

Familial aggregation by specific lymphoma subtypes has been more difficult to estimate due to changes in classification over time and the need for very large studies. As shown in Table 38.1, a family history of a given NHL subtype was associated with an increased risk of concordant as well as some discordant subtypes. Concordant associations were strongest for LPL/WM (RR = 25), MCL (RR = 9.0), CLL (RR = 8.5), and FL (RR = 2.1), but discordant associations of similar or only slightly weaker effect size were also commonly observed [235]. In contrast, DLBCL risk was stronger for a first-degree relative with LPL/WM (RR = 3.5) or FL (RR = 2.6) than for DLBCL (RR = 1.9). There are very limited data on PTCL, and registry-based studies suggested no increased risk among first-degree relatives with HL, CLL, DLBCL, or FL [237]. Similar to NHL subtypes, familial risks were elevated for both concordant and discordant HL subtypes, and some of the strongest associations were for concordant subtypes, including lymphocyte-rich (SIR = 81, 95% CI 30–177), lymphocyte-depleted (SIR = 40, 95% CI 1–225), mixed cellularity (SIR = 5.0, 95% CI 0.6–18), and nodular sclerosis (SIR = 4.6, 95% CI 2.9–7.0) [236]. While familial risk is determined by both shared genetics and environment, there was no increase in risk of concordant NHL, leukemia, HL, or MM among spouses [238], suggesting that familial risk is less likely to be associated with the environment shared during adulthood.

Case-Control and Cohort Studies

In a pooled analysis of 17,471 NHL cases and 23,096 controls from 20 case-control studies in the International Lymphoma Epidemiology (InterLymph) Consortium [239], risk of NHL (which included CLL) was associated with a family history of NHL (OR = 1.8), CLL (OR = 1.5), HL (OR = 1.7), and MM (OR = 1.8) (Table 38.1). The InterLymph analysis also reported risks for the common subtypes of CLL, DLBCL, FL, MCL, MZL, LPL/WM, and PTCL. A family history of NHL was associated with each subtype except LPL/WM, while a family history of leukemia was associated with each subtype except DLBCL and FL, with the strongest association for risk of CLL (OR = 2.4). A family history of HL was only associated with increased risk of DLBCL (OR = 2.1) and MZL (OR = 2.7) while a family history of MM was associated with FL (OR = 1.9), MCL (OR = 3.1), and PTCL (OR = 2.9).

Next, in an analysis of all subtypes simultaneously, there was no statistically significant heterogeneity across risk of most common NHL subtypes for either a family history of NHL ($P_{\text{Homogeneity}} = 0.52$) or HL ($P_{\text{Homogeneity}} = 0.47$) [239]. In contrast, there was strong evidence for heterogeneity for a family history of leukemia ($P_{\text{Homogeneity}} = 3.9 \times 10^{-5}$), with family history of leukemia most strongly associated with risk of CLL, LPL/WM, MCL, and PTCL. Also, there was modest evidence for heterogeneity for a family history of MM ($P_{\text{Homogeneity}} = 0.022$), with a family history of MM most strongly associated with MF/SS. Of note, the associations for family history of NHL with risk of NHL [239] or specific NHL subtypes (e.g., DLBCL [240], FL [241], CLL [242], MCL [243], MZL [244], LPL/WM [245], and PTCL [246]) remained unchanged after adjusting for extensive subtype-specific risk factors, suggesting that the association of family history may be predominately driven by shared genetics over shared environment.

While InterLymph did not have pooled results for risk of HL or MM, a large case-control study conducted in

Table 38.1 Risk[a] of lymphoma subtypes by family history of selected cancers in first-degree relatives

		Risk of selected lymphoma subtype									
Family history	Study design	NHL	CLL	HL	MM	DLBCL	FL	MCL	MZL	LPL/WM	PTCL
NHL	Registry [231, 235]	1.7 (1.4–2.2)	1.3 (0.9–1.9)	1.4 (1.0–2.0)	1.1 (0.8–1.5)	1.9 (1.6–2.2)	1.8 (1.5–2.1)	1.5 (1.0–2.3)	1.6 (0.8–2.8)	2.2 (1.5–3.2)	–
	Case-control [239, 247, 250]	1.8 (1.5–2.1)	1.9 (1.4–2.6)	3.3 (1.3–8.0)	1.4 (0.9–2.0)	1.8 (1.5–2.3)	2.0 (1.6–2.5)	2.0 (1.1–3.3)	1.7 (1.1–2.5)	1.2 (0.5–2.8)	1.7 (0.9–3.1)
CLL[b]	Registry [232]	1.9 (1.5–2.3)	8.5 (6.1–12)	1.5 (1.0–2.3)	1.2 (0.9–1.8)	1.0 (0.4–2.5)	1.6 (0.9–2.8)	1.1 (0.3–5.5)	–	4.0 (2.0–8.2)	1.3 (0.4–4.9)
	Case-control [239, 247, 250]	1.5 (1.3–1.8)	2.4 (1.9–3.1)	6.3 (1.3–30)	1.2 (0.9–1.6)	1.2 (0.9–1.5)	1.0 (0.7–1.3)	2.0 (1.2–3.2)	1.7 (1.2–2.4)	2.2 (1.2–4.0)	1.8 (1.1–3.1)
HL	Registry [233, 237]	1.3 (0.9–1.8)	2.1 (1.2–3.8)	3.1 (1.8–5.3)	1.0 (0.6–1.8)	2.4 ($P < 0.05$)	1.4 ($P > 0.05$)	–	–	–	No increase[c]
	Case-control [239, 247, 250]	1.7 (1.2–2.3)	1.3 (0.6–2.6)	3.3 (0.5–22)	1.0 (0.5–1.8)	2.1 (1.4–3.2)	1.5 (0.9–2.4)	1.5 (0.5–5.0)	2.7 (1.4–5.5)	2.2 (0.5–9.4)	0.6 (0.1–4.4)
MM	Registry [234]	1.1 (0.9–1.4)	1.1 (0.8–1.7)	0.9 (0.6–1.4)	2.1 (1.6–2.9)	–	–	–	–	1.4 (0.7–2.8)	–
	Case-control [239, 247, 250]	1.8 (1.2–2.7)	2.0 (0.9–4.3)	1.7 (0.3–8.8)	1.9 (1.3–2.9)	1.4 (0.7–2.6)	1.9 (1.1–3.5)	3.1 (1.1–9.1)	0.6 (0.1–2.4)	–	2.9 (1.0–8.4)
DLBCL	Registry [235, 237]	1.4 (1.2–1.6)	–	2.0 (1.1–4.0)	–	1.9 (1.4–2.6)	1.8 (1.2–2.7)	1.1 (0.3–2.9)	4.0 (1.5–8.7)	2.0 (0.7–4.4)	No increase[c]
	Case-control	–	–	–	–	–	–	–	–	–	–
FL	Registry [235, 237]	1.6 (1.4–2.0)	–	1.4 (NS)	–	2.6 (1.7–3.6)	2.1 (1.3–3.4)	2.6 (0.9–6.1)	1.1 (0.0–5.9)	1.8 (0.4–5.2)	No increase[c]
	Case-control	–	–	–	–	–	–	–	–	–	–
MCL	Registry [235]	1.5 (1.0–2.3)	–	–	–	1.9 (0.5–4.8)	2.0 (0.4–5.9)	9.0 (1.9–26)	–	3.5 (0.1–19)	–
	Case-control	–	–	–	–	–	–	–	–	–	–
LPL/WM	Registry [235, 370]	2.3 (1.5–3.4)	3.4 (1.7–6.6)	0.8 (0.3–2.2)	1.6 (0.8–3.2)	3.5 (1.3–7.5)	1.6 (0.2–5.9)	3.7 (0.1–21)	–	25 (9.1–54)	–
	Case-control	–	–	–	–	–	–	–	–	–	–

[a]Odds ratios (for case-control design) or relative risk (for registry design) and 95% confidence intervals
[b]CLL for registry studies; leukemia for case-control
[c]Estimate not reported

Scandinavia [247] reported elevated risk of HL with a family history of HL (OR = 3.3), NHL (OR = 3.3), CLL (OR = 6.3), and more weakly with MM (OR = 1.7); some smaller studies have reported larger ORs for risk of HL with a family history of HL [248, 249]. In a pooled analysis of 2843 cases and 11,470 controls from 11 studies, an elevated risk of MM was observed with a family history of MM (OR = 1.9), but no significant associations with NHL (OR = 1.4), leukemia (OR = 1.2), and HL (OR = 1.0) [250]. For blacks, the association for family history of MM was much stronger (OR = 5.5).

There are few cohort studies that have had detailed data on lymphoma in family members or a sufficient number of lymphoma outcomes to assess risk of specific NHL subtypes. In a national cohort study of 3.5 million people in Sweden born 1973–2008, family history of HL in a parent or sibling was associated with an 7.2 and 8.8-fold higher risk of childhood/young adult HL, respectively [251], while another study reported a sixfold higher risk for siblings [252], both much stronger than the associations shown in Table 38.1, perhaps due to the focus on childhood and young adult HL (and suggesting a stronger genetic predisposition in younger onset HL). In a cohort study of over 120,000 female California teachers [253], a history of lymphoma in a first-degree relative was associated with a 1.7-fold higher risk of B-cell NHL (RR = 1.74, 95% CI 1.16–2.60) based on 478 cases; data on risk for NHL subtypes were not available. This finding was highly consistent with pooled case-control data (Table 38.1).

Summary

Most of the risk estimates from population-based registry studies in Table 38.1 are broadly consistent with the estimates from case-control studies, with the most notable exception for a family history of MM with risk of NHL, CLL, and HL, where case-control studies show weak positive associations while the registry studies show no association. Taken together, these data provide strong evidence for familial predisposition across lymphoma subtypes, with the exception of MM. Further, familial risk does not seem to be confounded by nongenetic risk factors, although there are likely unidentified risk factors and clustering of known (and unknown) risk factors within families is difficult to exclude. This suggests a role for shared genetics underlying the familial aggregation. Although not universal, there is also clear evidence that family history of a specific lymphoma subtype is most strongly associated with risk for that specific lymphoma, suggesting that there may be some genetic factors unique to a subtype.

Prognosis

In contrast to etiology, there are relatively few data on the role of familial aggregation in NHL prognosis. The most comprehensive data are from a study using population-based registries in Sweden and Denmark that identified 41,026 patients with lymphoma, of whom 206 patients with NHL, 85 patients with CLL, and 95 patients with HL had a family history of any lymphoma [254]. Compared to patients without a family history of lymphoma, 5-year mortality for patients with a family history of lymphoma was similar for NHL (HR = 0.91, 95% CI 0.74–1.12), CLL (HR = 1.28, 95% CI 0.95–1.72), and HL (HR = 0.78, 95% CI 0.49–1.25), and these results were similar for 10-year mortality. NHL subtype was available on 28% of the cases, and there were no associations of family history with survival for high grade B-cell or low grade B-cell lymphoma, while there was a suggestive association for T-cell/anaplastic lymphoma (HR = 5.38, 95% CI 1.65–17.5). In other analyses limited to the Swedish registries, there was no difference in cause-specific or overall survival for patients with sporadic NHL compared to patients with a parent or offspring with NHL [255, 256] or lymphatic leukemia [256]. In an Italian clinical registry, patients with sporadic CLL had a similar rate of requiring therapy and 10-year survival as CLL patients with a family history of any hematologic malignancy or a first-degree relative with CLL [257]. Using cases enrolled in a population-based case-control study from Sweden [258], a family history of hematologic malignancy was not associated with all-cause or cause-specific survival after DLBCL, FL, MCL, or T-cell lymphoma. In summary, the current literature, although not extensive, suggests that familial aggregation does not play a major role in the prognosis of NHL or the most common NHL subtypes.

Host (Germline) Genetics

While the studies of familial aggregation are consistent with an important role for genetics in lymphoma development (but perhaps not prognosis), they cannot definitively separate shared genetics from shared environment. We next review studies that show not only clear evidence of a genetic contribution to lymphoma risk and prognosis, but also provide chromosomal locations that are associated with risk.

Etiology

Family Genetic Linkage Studies

A limited number of lymphoma family studies have been conducted to identify genes with major susceptibility effect. These studies are based on the idea that related individuals who are affected with lymphoma have a shared genetic exposure. Linkage studies in HL have identified both HLA class I (for EBV+) and class II (for EBV−) risk and protective alleles and haplotypes [259, 260]. Beyond HLA, linkage

studies in CLL [261], HL [262], and WM [263] have not definitively identified genes with large effects, and there are no published studies in FL, DLBCL, or other NHL subtypes. For CLL, significant linkage was localized to 2q21.2, which contains the chemokine receptor (*CXCR4*) gene and for which rare coding mutations have been identified [264]. Using more recent NGS technology, additional family studies of WM [265], HL [266, 267], and CLL [268, 269] have been done. These studies have identified rare variants in a small number of families, but still need further validation. A lack of strong findings for these family studies may be due to small sample sizes or the relatively private nature of these rare variants segregating in only a few families, but also raises the hypothesis that multiple, low to moderate risk variants that are common in the population, defined as minor allele frequency (MAF) >5%, may be more relevant in lymphoma etiology than single, highly penetrant variants that are very rare.

Candidate Gene Studies

The choice of a candidate gene or pathway has been mainly driven by a priori biologic knowledge of lymphoma and conditions associated with lymphoma such as autoimmune and infectious diseases as well as other cancers, and have been reviewed extensively [260, 270–274]. Most findings from these studies have failed to replicate, likely due to issues related to study design, bias from population stratification (i.e., confounding by race or ethnicity), small sample size (low power), uncontrolled multiple testing (leading to false positive associations), and unrealistic expectations in our ability to choose variants and genes [275]. The most robust findings have been for an *LTA-TNF* haplotype with DLBCL ($P = 2.93 \times 10^{-8}$) [276, 277]; a SNP (rs3789068) in the *BCL2L11* and risk for B-cell NHL (OR = 1.21, $P = 2.21 \times 10^{-11}$) [278]; a SNP (rs2266690) in *CCNH* with CLL risk (OR = 0.63, $P = 1.62 \times 10^{-8}$) [279]; a SNP (rs3132453) in *PRRC2A* in HLA class III and risk of B-cell NHL (OR = 0.68, $P = 1.07 \times 10^{-9}$) [278]; and certain HLA alleles in class I (including *HLA-A*01* and **02*) with EBV+ HL and class II (including *HLA-DRB1*) with EBV− HL [260].

GWAS

In contrast to candidate gene/pathway studies, GWA studies have definitively identified multiple susceptibility loci for lymphoma, which are summarized in Table 38.2. This success is in part due to the stringent level of evidence (currently $P < 5 \times 10^{-8}$) and independent replication across multiple independent studies. Most GWAS has also been based on specific lymphoma subtypes, with only one GWAS based on all lymphomas (which included HL, MM, and other NHL subtypes) in both discovery and validation stages, and a SNP at 11q12.1 (near *LPXN*) was identified, and the associations were consistent (but not genome-wide significant) in analysis of subtypes [280]. However, this locus has not been replicated in larger GWAS studies based on specific subtypes.

CLL

The estimated contribution of all common variation to the heritability of CLL is 46–59% [281, 282]. The first GWAS in a lymphoid malignancy was conducted for CLL [283] and to date, GWAS analyses [281, 284–290] have identified 45 SNPs from 39 loci for CLL, which accounts for approximately 25% of familial risk of CLL [290]. Many of these GWAS SNPs are near or in genes plausibly linked to CLL, including genes involved in apoptosis (including *FAS, PMAIP1, BAK1, BCL2, BCL2L11, BMF, CASP8/CASP10*), telomere function (*POT1, TERT, TERC*), transcription factors important in B-cell differentiation (*IRF8, LEF1, PRKD3, SP140*), and B-cell receptor activation (*IRF3, HLA-DQA1*). Although there has been little evidence of statistical interaction among these GWAS SNPs, there is clear evidence of biological interaction among the proteins of the genes in proximity to the GWAS SNPs [290]. None of the GWAS SNPs have individually shown a strong relationship with age at diagnosis, although cases diagnosed at a younger age tended to carry a greater number of risk alleles [287], supporting the hypothesis that early onset CLL is enriched for genetic susceptibility.

In an East Asian population of 71 CLL cases and 1273 controls, GWAS-discovered SNPs for CLL near *IRF4* (rs872071), *SP140* (rs13397985), and *ACOXL* (rs17483466) were associated with CLL risk at nominal significant level ($P < 0.05$), with a suggestive association with *GRAMD1B* (rs735665) [291]. The minor allele frequencies of these SNPs were much lower than in populations of European descent, supporting the hypothesis that the lower prevalence of CLL genetic risk factors might explain part of the lower incidence of CLL in East Asian populations. In a separate study of 117 Mexican mestizos CLL cases, the rs872071 SNP in *IRF4* was found to be statistically associated with CLL risk with similar minor allele frequencies as that of Caucasians. Finally, in a study of 110 African American CLL cases, none of the 15 evaluated GWAS SNPs was found to be associated with CLL risk at nominal level of statistical significance ($P < 0.05$).

Hodgkin Lymphoma

Classical HL (cHL) makes up approximately 95% of HL, and cHL compromises several subtypes: in young children and older adults, mixed cellularity HL (typically EBV+) predominates, while in adolescents and young adults, nodular sclerosing HL (typically EBV−) predominates [292]. Five GWAS analyses have been published in HL [293–297], and the strongest findings have been for SNPs mapping to HLA class II [293–295] in close proximity to *HLA-DRA* and

Table 38.2 GWAS-discovered loci for lymphoma

Subtype	Locus	SNP	Nearest Gene	RAF[a] (controls)	OR[b]	*P*-value[c]	References
CLL	1p36.11	rs34676223	*MDS2*	0.71	1.19	5.04×10^{-13}	[290]
CLL	1q42.13	rs41271473	*RHOU*	0.79	1.19	1.06×10^{-10}	[290]
CLL	2p22.2	rs3770745	*QPCT, PRKD3*	0.22	1.24	1.68×10^{-8}	[281]
CLL	2q13	rs13401811	*ACOXL, BCL2L11*	0.81	1.41	2.08×10^{-18}	[281]
CLL	2q13	rs17483466	*ACOXL, BCL2L11*	0.20	1.39	2.36×10^{-10}	[283]
CLL	2q13	rs9308731	*ACOXL, BCL2L11*	0.54	1.19	1.00×10^{-11}	[289]
CLL	2q33.1	rs3769825	*CASP10/CASP8*	0.45	1.19	2.50×10^{-9}	[281]
CLL	2q37.1	rs13397985	*SP140, SP110*	0.19	1.41	5.40×10^{-10}	[283]
CLL	2q37.3	rs757978	*FARP2*	0.11	1.39	2.11×10^{-9}	[371]
CLL	3p24.1	rs9880772	*EOMES*	0.47	1.19	2.55×10^{-11}	[289]
CLL	3q26.2	rs10936599	*MYNN*	0.75	1.26	1.74×10^{-9}	[287]
CLL	3q28	rs9815073	*LPP*	0.65	1.18	3.62×10^{-8}	[289]
CLL	4q24	rs71597109	*BANK1*	0.69	1.17	1.37×10^{-10}	[290]
CLL	4q25	rs898518	*LEF1*	0.59	1.20	4.24×10^{-10}	[281]
CLL	4q26	rs6858698	*CAMK2D*	0.16	1.31	3.07×10^{-9}	[287]
CLL	4q35.1	rs57214277	*IRF2*	0.41	1.13	3.69×10^{-8}	[290]
CLL	5p15.33	rs10069690	*TERT*	0.25	1.20	1.12×10^{-10}	[287]
CLL	6p25.3	rs872071	*IRF4*	0.54	1.54	1.9×10^{-20}	[283]
CLL	6p25.2	rs73718779	*SERPINB6*	0.11	1.26	1.97×10^{-8}	[289]
CLL	6p21.32	rs9273363	*HLA-DQB1*	0.27	1.24	2.24×10^{-10}	[281]
CLL	6p21.32	rs674313	*HLA-DRB1*	0.26	1.69	6.92×10^{-9}	[285]
CLL	6p21.31	rs210142	*BAK1*	0.70	1.40	9.47×10^{-16}	[286]
CLL	6p21.31	rs3800461	*C6orf106*	0.11	1.20	1.97×10^{-8}	[290]
CLL	6q25.2	rs2236256	*IPCEF1*	0.44	1.23	1.50×10^{-10}	[287]
CLL	7q31.33	rs17246404	*POT1*	0.71	1.22	3.40×10^{-8}	[287]
CLL	8q22.3	rs2511714	*ODF1*	0.41	1.16	2.90×10^{-9}	[287]
CLL	8q24.21	rs2456449	*CASC19, POU5F1B*	0.36	1.26	7.84×10^{-10}	[371]
CLL	9p21.3	rs1679013	*CDKN2B-AS1*	0.52	1.19	1.27×10^{-8}	[281]
CLL	10q23.31	rs4406737	*ACTA2, FAS*	0.57	1.27	$1.22 \times \times 10^{-14}$	[281]
CLL	11p15.5	rs7944004	*C11orf21*	0.49	1.20	2.15×10^{-10}	[281]
CLL	11q23.2	rs61904987	*USP28, TMPRSS5*	0.12	1.24	2.46×10^{-11}	[290]
CLL	11q24.1	rs735665	*GRAMD1B*	0.21	1.45	3.78×10^{-12}	[283]
CLL	12q24.13	rs10735079	*OAS3*	0.36	1.18	2.34×10^{-8}	[288]
CLL	15q15.1	rs8024033	*BMF, BUB1B*	0.51	1.22	2.71×10^{-10}	[281]
CLL	15q21.3	rs7169431	*RFX7, NEDD4*	0.08	1.36	4.74×10^{-7c}	[284]
CLL	15q23	rs7176508	*RPLP1*	0.37	1.37	4.54×10^{-12}	[283]
CLL	16q24.1	rs305061	*IRF8*	0.66	1.22	3.60×10^{-7c}	[284]
CLL	16q24.1	rs2292982	*IRF8*	0.34	0.65	6.48×10^{-9}	[285]
CLL	18q21.1	rs1036935	*CXXC1*	0.22	1.15	3.27×10^{-8}	[290]
CLL	18q21.32	rs4368253	*PMAIP1*	0.69	1.19	2.51×10^{-8}	[281]
CLL	18q21.33	rs4987855	*BCL2*	0.91	1.47	2.66×10^{-12}	[281]
CLL	18q21.33	rs4987852	*BCL2*	0.06	1.41	7.76×10^{-11}	[281]
CLL	19p13.3	rs7254272	*ZBTB7A*	0.18	1.17	4.67×10^{-8}	[290]
CLL	19q13.32	rs11083846	*PRKD2*	0.22	1.35	3.96×10^{-9}	[283]
CLL	22q13.33	rs140522	*NCAPH2*	0.32	1.15	2.70×10^{-9}	[290]
DLBCL	2p23.3	rs79480871	*NCOA1*	0.076	1.34	$4.23 \times \times 10^{-8}$	[309]
DLBCL	3q27	rs6773854	*BCL6/LPP*	0.22	1.47	1.14×10^{-11}	[311]
DLBCL	6p21.33	rs2523607	*HLA-B*	0.12	1.32	2.40×10^{-10}	[309]
DLBCL	6p25.3	rs116446171	*EXOC2*	0.019	2.20	2.33×10^{-21}	[309]
DLBCL	8q24.21	rs13255292	*PVT1*	0.32	1.22	9.98×10^{-13}	[309]

(continued)

Table 38.2 (continued)

Subtype	Locus	SNP	Nearest Gene	RAF[a] (controls)	OR[b]	*P*-value[c]	References
DLBCL	8q24.21	rs4733601	*PVT1*	0.48	1.18	3.63×10^{-11}	[309]
FL	3q28	rs6444305	*LPP*	0.27	1.21	1.10×10^{-10}	[308]
FL	6p21.32	rs10484561	MHC class II	0.13	1.95	1.12×10^{-29}	[50]
FL	6p21.32	rs2647012	*HLA-DQB1*	0.44	0.64	2.00×10^{-21}	[307]
FL	6p21.32		HLA-DRβ1 Glu-28	0.3	1.86	7.89×10^{-69}	[308]
FL	6p21.32	rs17203612	*HLA-DRA*	0.49	1.44	4.59×10^{-16}	[308]
FL	6p21.33	rs3130437	*HLA-C*	0.62	1.23	8.23×10^{-9}	[308]
FL	6p21.33	rs6457327	*C6orf15* et al. *(STG)*	0.38	0.59	4.70×10^{-11}	[306]
FL	8q24.21	rs13254990	*PVT1*	0.32	1.18	1.06×10^{-8}	[308]
FL	11q23.3	rs4938573	*CXCR5*	0.20	1.34	5.79×10^{-20}	[308]
FL	11q24.3	rs4937362	*ETS1*	0.46	1.19	6.76×10^{-11}	[308]
FL	18q21.33	rs17749561	*BCL2*	0.91	1.34	8.28×10^{-10}	[308]
HL	2p16.1	rs1432295	*REL*	0.40	1.22	1.91×10^{-8}	[294]
HL	3p24.1	rs3806624	*EOMES*	0.45	1.26	1.14×10^{-12}	[297]
Hl (EBV−)	5q31	rs20541	*IL13*	0.18	1.37	1.00×10^{-10}	[293]
HL	6p21	rs2248462	*MICB*	0.22	0.61	1.30×10^{-13}	[293]
HL	6p21	rs2395185	*HLA-DRA*	0.33	0.56	8.30×10^{-25}	[293]
Hl (EBV+)	6p21	rs2734986	*HLA-A*	0.18	2.45	1.20×10^{-15}	[293]
Hl (EBV+)	6p21	rs6904029	*HCG9*	0.30	0.46	5.50×10^{-10}	[293]
HL	6p21.32	rs2281389	*HLA-DRA*	0.83	1.73	6.31×10^{-13}	[300]
HL	6p21.32	rs6903608	*HLA-DRA*	0.27	1.7	2.84×10^{-50}	[294]
HL (NS)	6p21.32	rs2858870	*HLA class II*	0.13	0.40	5.61×10^{-9}	[295]
HL (NS)	6p21.32	rs204999	*HLA class II*	0.27	0.60	2.34×10^{-8}	[295]
HL	6q23.3	rs7745098	*HBS1L, MYB*	0.48	1.21	3.42×10^{-9}	[297]
HL	8q24.21	rs2019960	*PVT1*	0.23	1.33	1.26×10^{-13}	[294]
HL	10p14	rs501764	*GATA3*	0.19	1.25	7.05×10^{-8}	[294]
HL	19p13.3	rs1860661	*TCF3*	0.41	0.81	3.50×10^{-10}	[296]
LYM	11q12.1	rs12289961	*LPXN*	0.28	1.29	3.89×10^{-8}	[280]
MZL	6p21.32	rs9461741	*BTNL2*	0.018	2.66	3.95×10^{-15}	[312]
MZL	6p21.33	rs2922994	*HLA-B*	0.11	1.64	2.43×10^{-9}	[312]
NKTCL	6p21.32	rs9277378	*HLA-DPB1*	0.29	1.84	4.21×10^{-9}	[313]

[a]Risk allele frequency among controls
[b]Odds Ratio (per allele)
[c]Considered genome-wide significant at the time of initial publication

HLA-DRB1, regions previously linked to HL by HLA-typing studies [298, 299]. The 6p21.32 locus marked by rs6903608 (near *HLA-DRA*), was associated with cHL overall and more specifically to EBV-negative cHL [293, 294], early onset [294, 300] and young adult nodular sclerosing HL (largely EBV−) [295]. Additional GWAS signals at 6p21 have been identified in HLA class I [293], with statistically independent associations for rs2248462 (near *MICB*) for all cHL (irrespective of EBV status), and rs2734986 (3′ untranslated region of *HLA-G* and near *HLA-A*) and rs6904029 (near *HCG9*) for EBV+ cHL. These results confirm earlier studies linking *HLA-A*01* and **02* to EBV+ cHL [301–303] and support a role for class I but not class II genes in EBV+ HL. Using SNPs to impute classic HLA alleles, two independent signals in the HLA class II region (rs6903608 and rs2281389) were linked to early onset HL, but no specific classical HLA alleles from this region were significant after conditioning on these two SNPs [300]. The class II SNP rs6903608 was estimated to account for ~6% of the familial risk in HL [300].

Outside of the HLA complex, GWAS-discovered loci for HL include 2p16.1 [294] (near *REL*), 10p14 (near *GATA3*) [294], 8q24.21 (telomeric to *PVT1* and near *MYC*) [294], 5q31 (a nonsynonymous SNP in *IL13*) [293], 3p24.1 (5′ to *EOMES*) [297], 6q23.3 (intergenic to *HBS1L* and *MYB*) [297], and 19p13.3 (in intron 2 of *TCF3*) [296], with only the 2p16.1 and 5q31 loci showing stronger associations with EBV (negative) status. Genes from these non-HLA regions are involved in hematopoiesis and immunoregulation, making them plausible susceptibility loci for cHL. HLA and non-HLA-linked loci appear to be independent, and non-HLA loci were estimated to account for ~7% of the familial risk in

HL [297]. A recent comparison of heritability estimates on using a genomic versus a population approach supported an underlying polygenic basis for HL susceptibility and that there were likely additional loci outside of chromosome 6 (HLA) remain to be detected [304].

These studies have been mainly conducted in studies of European ancestry. In a study of cHL in a northern Chinese population [305], HLA-A*02:07 was a predisposing allele for EBV+ cHL and a protective allele for EBV− cHL, and this allele was more common in the Chinese vs. European populations, emphasizing the need for more studies in diverse populations.

Follicular Lymphoma

Three early GWA studies based on small discovery sets (<400 cases) identified loci at 6p21.33 [306] and 6p21.32 [50, 307] in the HLA region associated with FL. In a meta-analysis of those studies plus a new GWAS of over 2100 cases, the HLA region showed overwhelming association with FL, with over 8000 SNPs achieving genome-wide significance. After additional validation, a top SNP from this region (rs12195582) reached $P = 5.35 \times 10^{-100}$ [308]. HLA alleles and amino acids (AA) were imputed and the top signal mapped to four linked DRβ1 multi-allelic AA at positions 11, 13, 28, and 30, suggesting an important role for DRβ1 peptide presentation in FL [308]. Additional independent signals were also identified in HLA class II (rs17203612) and class I (rs3130437, near *HLA-C*); after accounting for all of these signals, no other previously identified SNP from the HLA region achieved genome-wide significance. Outside of the HLA region, five novel loci have been identified including 11q23.3 (near *CXCR5*), 11q24.3 (near *ETS1*), 3q28 (in *LPP*), 18q21.33 (near *BCL2*), and 8q24 (near *PVT1*) [308]. These genes are linked to B-cell biology making them plausible in the etiology of FL.

DLBCL

In a large GWAS of European ancestry [309], novel loci identified included 6p25.3 (*EXOC2*), 6p21.33 (*HLA-B*), 2p23.3 (*NCOA1*), and 8q24.21 (near *PVT1* and *MYC*); the strongest finding after imputing HLA alleles and AA was with *HLA-B*08:01*, although this could not be statistically distinguished from the *HLA-B* SNP due to high LD. That study further estimated that common SNPs, including but not limited to the GWAS-discovered loci, explained approximately 16% of the variance in DLBCL risk overall. To date, no GWAS study has published DLBCL results by cell-of-origin (i.e., germinal center versus activated B-cell).

Three of the five GWAS-discovered SNPs for DLBCL in Europeans were significantly associated with DLBCL in an East Asian population [310], including *EXOC2* (OR = 2.04, $P = 3.9 \times 10^{-10}$), *PVT1* (OR = 1.34, $P = 2.1 \times 10^{-6}$), and *HLA-B* (OR = 3.05, $P = 0.009$). Overall, MAFs were similar or only modestly lower in the East Asian population for all SNPs except for one of the 8q24 SNPs, which was much rarer. In a GWAS conducted in an East Asian population, a locus at 3q27 (near *BCL6* and *LPP*) was identified [311], although this could not be replicated in independent studies of East Asian [310] or European ancestry [309].

Other Subtypes

A GWAS of MZL [312] identified two distinct loci at 6p21.32 (intragenic to *BTNL2*, in HLA class II) and 6p21.33 (*HLA-B*, in HLA class I); these two loci were in low LD and were statistically independent of each other. There was no strong heterogeneity in these results when stratified on MALT versus non-MALT (SMZL and NMZL) subtypes, although this was based on a modest sample size. These loci have been associated with autoimmune diseases and immune response, suggesting shared biologic underpinnings with MZL. A GWAS of extranodal natural kill T-cell lymphoma (NKTCL) in Asians identified a locus at 6p21.32 (*HLA-DPB1* in HLA class II), and imputation-based fine-mapping implicated four AA residues (Gly84-Gly85-Pro86-Met87) at the edge of the HLA-DPB1 peptide-binding groove as accounting for most of the association of the top SNP in this region. Furthermore, none of the other published FL, DLBCL, or MZL GWAS SNPs was associated with NKTCL [313]. While NKTCL in Asia has been linked to EBV, the genetic associations are distinct from those in EBV+ HL (see above).

Gene × Environment Interaction

Given the lack of validated candidate genes, there have been even fewer robust studies of gene–environment interaction. The most robust results are from large pooled studies, which suggest an interaction of B-cell-mediated autoimmune conditions and the *TNF* SNP rs1800629 with NHL risk [314] and body mass index and the *TNF* SNP rs1800629 with DLCBL risk [315]. A large pooled analysis found no association of current cigarette smoking and genetic variation in N-acetyltransferase enzymes and FL risk [316].

Summary

To date, GWAS have successfully identified 78 SNPs from 65 genetic loci, mainly associated with specific subtypes (Fig. 38.3), with only three regions—the HLA region, 8q24, and 3p24.1—associated with multiple lymphoma subtypes. However, even the associations for the common regions are heterogeneous, particularly for HLA [274]. The established SNPs are common (MAF > 5%) and generally have small effect sizes (ORs 0.6 to 2.0). In contrast to GWAS, candidate gene studies in lymphoma have had only minimal success, similar to other cancers [317]. Linkage studies have also not been successful in identifying rare alleles causing Mendelian disease, and the evaluation of low-frequency

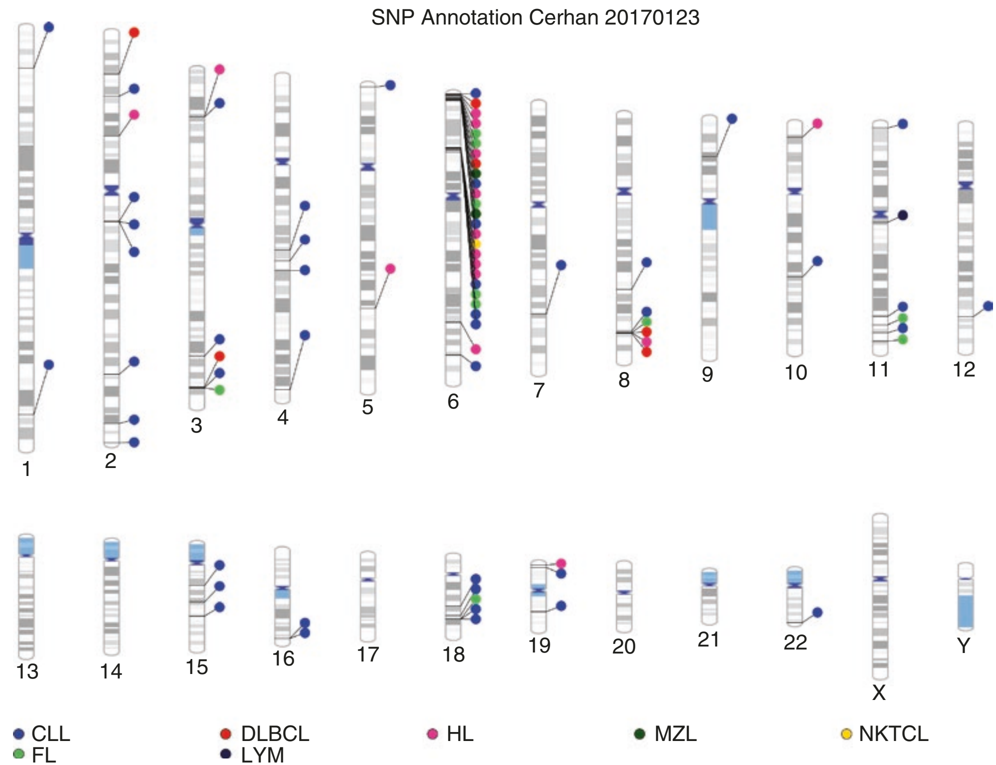

Fig. 38.3 GWAS-discovered loci for lymphoma subtypes mapped to chromosomal location. Except for 6p21, 8q24, and 3p24.1, most susceptibility loci are unique to only one lymphoma subtype

variants with intermediate effects is still in early research phases for lymphoma, but will be challenged by sample size issues [318]. The GWAS-identified SNPs that have been identified are largely of unknown function, and this is a major task in the "post-GWAS" era. One leading hypothesis on the mechanistic role of these common SNPs is their effect on gene expression (e.g., through effects on promoters or enhancers), but this effect is difficult to identify given an expected modest impact of these SNPs on gene expression and the fact that this impact could occur at any time before diagnosis [287].

Prognosis

Candidate Gene/Pathway Studies

Germline genetics as predictors of outcome after a diagnosis of lymphoma, including treatment response, toxicity (e.g., neutropenia and cardiotoxicity), event-free (EFS)/PFS, and OS are still relatively limited. Most have been candidate gene/pathway studies, with a focus on immunologic and metabolism (particularly drug metabolism) pathways, as well as downstream mechanisms of action of treatments including reactive oxygen species (ROS) and DNA repair; there have only been two published GWAS analyses, both of which were re-purposed from etiology studies. We review the most robust findings based on larger studies and replication in at least one independent study.

Cytokine Genes

The first report in the literature on germline SNPs and prognosis in lymphoma were from genes encoding the pro-inflammatory cytokines tumor necrosis factor (TNF) and lymphotoxin (LT)-α. Specifically, a long-range haplotype consisting of alleles associated with higher gene expression from *TNF* (rs1800629, A allele) and *LTA* (rs909253, A allele) were associated with shorter PFS (HR = 2.33, $P = 0.0053$) and OS (HR = 1.92, $P = 0.081$) in DLBCL after adjustment for the International Prognostic Index (IPI), while there was no association with FL [319]. The positive findings for DLBCL [320, 321] and null findings for FL [322, 323] were confirmed in other pre-rituximab era patient cohorts, while in rituximab-treated DLBCL patients there was no association for either *TNF* (HR = 0.94, $P = 0.79$) or *LTA* (HR = 0.90, $P = 0.66$) [321], highlighting that changes in treatment can impact prognostic associations, as reported for some tumor markers in the rituximab era [324]. In CLL, the *TNF* SNP was associated with OS after adjustment for Rai stage and IGHV mutation status ($HR_{AA/AG} = 2.1$, $P = 0.01$), but was not associated with treatment-free survival [325]. The *TNF* SNP was not associated with PFS in cHL overall, but in a meta-analysis of two cohorts, it was associated with PFS in EBV− (but not EBV+) cHL ($HR_{AA/AG} = 2.11$, $P = 0.01$) [326].

Only two other cytokine SNP markers in *IL8* and *CD46* have shown some promising results. IL-8 is a potent chemoattractant for neutrophils and monocytes, and the A allele of *IL8* rs4073 has been associated with increased production

of IL-8 [327] and inferior OS in a cohort of 278 FL patients ($HR_{AA/AT}$ = 0.47, P = 0.06) [323] and PFS (but not OS) in a cohort of 231 FL patients ($HR_{per\ A\ allele}$ = 0.78, P = 0.02) [328]; however, a smaller cohort of 117 FL patients found the opposite association for OS [329]. CD46 regulates complement and regulatory T-cell function, and the *CD46* rs2466571 SNP was associated with inferior EFS in a US ($HR_{CC\ vs.\ AA}$ = 1.96, P = 0.049) [330] and a Swedish ($HR_{per\ C\ allele}$ = 1.37, P = 0.006) cohort of FL patients [328], with the former study also finding a stronger association in patients who were observed. Other early leads, including associations with *IL10*, *IL8RB*, *IL1A* and *IL4* and *IL4R* in DLBCL [320, 321, 329] and *IL2*, *IL12B* and *IL1RN* in FL [323, 328], *IL10* and *IL6* in HL [326, 331, 332] as well as attempts at multi-gene modeling [320, 323, 328, 333], have yet to be replicated.

FCGR2A and *FCGR3A*

Preclinical studies suggest that SNPs in Fc gamma receptor (*FCGR*) genes could influence response to rituximab related to efficacy of Fc binding and triggering of antibody-dependent cell-mediated cytotoxicity [334]. Early studies of rituximab monotherapy for FL found that the *FCGR3A* (rs396991, V158F) VV genotype (compared to F carriers) and/or the *FCGR2A* (rs1801274, H131R) HH genotype (compared to R carriers) (the two genes are in LD [335]) was associated with better response rate and/or longer time to progression [336–338]. However, subsequent studies have been mixed [271], and a recent large prospective evaluation of this hypothesis embedded in the RESORT trial of treatment naïve, low tumor burden patients treated with R-monotherapy, found no evidence for either SNP with response or outcome [339]. In addition, neither SNP appears to predict response or prognosis in FL patients treated with CHOP [340, 341] or R-CHOP [342], including the large PRIMA trial [343], nor in patients treated for DLBCL [344, 345], HL [346], or CLL [347].

Pharmacogenetics and Metabolism

Interindividual variability in efficacy and toxicity of pharmacologic agents is affected in part by host genetic background in drug metabolism, detoxification, and transport and underlies the field of pharmacogenetics [348], but studies in lymphoma have been fairly limited. HL patients with the wild-type *UGT1A1* TA6/TA6 genotype (a phase 2 enzyme involved in the metabolism of anthracyclines and other xenobiotics) had inferior OS (HR = 2.54, P = 0.04) and freedom from progression (HR = 2.70, P = 0.004) compared to those with a least one *UGT1A1*28* allele in a cohort of 313 HL patients [349], and this was replicated for OS (HR = 4.20, P = 0.003) but not EFS (HR = 0.73, P = 0.25) in a cohort of 224 HL patients [350]. In a study of 106 R-CHOP21-treated DLBCL patients, the *CYBA* SNP rs4673 was associated with inferior EFS (HR_{TT} = 2.06, P = 0.038) after adjusting for IPI [351]. While the latter association was not replicated in a study of aggressive NHL which used a discovery (N = 401) and validation (N = 477) design [352], another *CYBA* SNP, rs1049255, was associated with inferior EFS (HR_{GG} = 1.58, P = 0.00005) and OS (HR_{GG} = 1.38, P = 0.018). Further functional analysis showed that the G allele of *CYBA* rs1049255 was associated with lower NAD(P)H activity, mRNA and protein expression [352].

In DLBCL and other aggressive NHLs, the *NCF4* rs1883112 SNP A allele was associated with greater hematologic [351, 353] and anthracycline-induced cardiotoxicity [354], as well as with superior EFS in two independent studies (meta-RR_{AA} = 0.66, P < 0.01) [353], although this was not observed in two other studies [351, 352]. *CYBA* encodes a membrane-bound p22phox subunit and *NCF4* encodes a cytosolic p40phox subunit of the NAD(P)H oxidase complex. NAD(P)H is a multi-unit complex that generates reactive oxygen species (ROS), which is one of the antitumor mechanisms of doxorubicin [355]. Genetic variation in other NAD(P)H subunits (*CYBB*, *NCF2*, *RAC2*) have not been associated with DLBCL prognosis [351, 352]. While other genes encoding metabolic enzymes, including cytochrome p450 (e.g., *CYP2C19*, *CYP3A4, CYP3A5*), detoxification (e.g., *GSTA1, GSTM1, GSTP1*), ATP-binding cassette transporters (e.g., *ABCB1*, *ABCC2*, *ABCG2*) have been evaluated with outcomes in HL, FL, or DLBCL, none have been robustly replicated [260, 271, 351].

DNA and Chromosomal Integrity

DNA damage signaling and repair, as well as one-carbon metabolism pathways, have important functions in maintaining DNA and chromosomal integrity, and many of the chemotherapeutic agents used to treat lymphomas rely on DNA damage as part of their mechanism of action in tumor cell killing. Using a discovery (N = 163) and validation (N = 145) study design, the *MLH1* rs1799977 AG/GG genotype was associated with inferior OS (HR = 3.14, P < 0.001) and PFS (HR = 1.96, P = 0.010) after adjusting for IPI and other clinical factors in R-CHOP21-treated DLBCL patients, and these results replicated in the validation set [356]. Furthermore, DLBCL patients with a risk (G) allele had significantly lower MLH1 expression in their tumors, consistent with its role in triggering apoptosis induced by chemotherapy [357]. In a study of 215 CHOP-treated DLBCL patients from the pre-rituximab era, the *MLH1* SNP was only weakly associated with OS (HR = 1.56, 95% CI 0.89–2.72) [358]. Other SNPs from these pathways for DLBCL and FL outcomes have not replicated [356, 358].

GWAS

There are only two published GWAS of prognosis in lymphoma, and both have struggled to meet the genome-wide

significance level used in etiology studies ($P < 5 \times 10^{-8}$). In a multistage GWAS of immunochemotherapy-treated DLBCL ($N = 852$ discovery, $N = 623$ validation), rs7712513 at 5q23.2 (near *SNX2* and *SNCAIP*) was associated with EFS (HR = 1.39, $P = 2.08 \times 10^{-7}$) and OS (HR = 1.49, $P = 3.53 \times 10^{-8}$) and rs7765004 at 6q21 (near *MARCKS* and *HDAC2*) was associated with EFS (HR = 1.38, $P = 7.09 \times 10^{-7}$) and OS (HR = 1.47, $P = 5.36 \times 10^{-7}$) [359]. The study provided encouraging evidence for these novel loci, which were distinct from DLBCL risk loci discussed above, and suggested both novel biology and the potential contribution of host genetics to DLBCL prognosis. In a GWAS of 586 FL patients (treatments unknown), a locus at 17q24 near *ABCA10* and *ABCA6* (rs10491178 HR = 3.17, $P = 5.24 \times 10^{-8}$) was associated with lymphoma-specific survival, were distinct from the risk loci, and implicated genetic variation in multidrug resistance for FL outcome [328]. When GWAS-identified susceptibility loci for HL (*HLA-DRA*, *REL*, *MYC/PVT1*, *GATA3*, and *TCF3*) were evaluated for prognosis in a meta-analysis of two large cohort studies, only the *MYC/PVT1* SNP rs2608053 was associated with EFS (HR = 1.94, $P = 0.01$) and OS (HR = 1.80, $P = 0.04$), and this association remained after adjustment for the International Prognostic Score (IPS) [360].

GWAS can also be used to identify specific adverse outcomes. In a GWAS study of children with HL who were treated with radiation therapy, two variants (rs4946728 OR = 3.32, $P = 5.99 \times 10^{-10}$ and rs1040411 OR = 2.39, $P = 1.18 \times 10^{-7}$) at 6q21 were associated with risk of second malignant neoplasms [361]. The risk haplotype was associated with lower expression of *PRDM1* and impacted induction of the protein after radiation exposure.

Summary

Similar to etiology studies, most candidate gene studies of prognosis have failed to replicate, likely due to the same issues for case-control studies discussed above, with additional issues that many prognosis studies included use of heterogeneous patient populations (i.e., mix of de novo and relapsed cases; multiple subtypes mixed together), study designs (randomized trials vs. observational studies), treatments (e.g., often heterogeneous, and sometimes out of date), and study endpoints (e.g., EFS, lymphoma-specific survival, OS), further complicating interpretation of the literature. The prognostic significance may also change with changes in treatment over time. Overall, there is a need for larger studies with more homogeneous treatments and clinically relevant endpoints beyond just OS. While results have not been particularly robust to date and more studies are needed, one early and potentially important theme is that loci associated with lymphoma risk largely do not overlap the loci associated with prognosis.

Practice Implications

Genomics underlies much of the goal towards precision medicine [362, 363], including tumor detection, accurate diagnosis and characterization (precision oncology), choice of treatment ("targeted" therapy), response to therapy (pharmacogenomics), predictive and prognostic models, tumor monitoring, and other outcomes such as second cancers and other morbidities. Applying genomics for precision prevention is a related goal, both for high risk families and the general population [364].

Use of Tumor Genomic Data in the Clinic

Currently there are numerous clinical grade molecular diagnostic tests that can be used for identification of somatic mutations in clinical practice, such as FoundationOne® Heme. Additionally, mutation profiles are now being used as therapeutic targets and for design of clinical trials. While the field is moving rapidly and advances have been made, many unanswered questions remain, most importantly, we currently do not consider the entire genomic landscape (genome, transcriptome, epigenome) when making clinical decisions. Moving forward a key challenge for use of NGS data in clinical practice will be our ability to understand the significance of mutations alone or in combination, to gain insight on the biologic significance of mutations, and to gain a better understanding of which mutations drive tumor formation versus those that impact response to therapy and patient outcome. Bioinformatic and technologic advances will be key to moving forward. Finally, tumor genomic evaluation is often paired with germline evaluation, with rapidly evolving implications for patients and families of clinically actionable secondary germline findings [365].

Use of Host (Germline) Genetics in Risk Assessment

Given the estimated lifetime risk of NHL is 1 in 48 (2.1%) in the United States [366] and an RR of 1.7 for the risk of NHL in a first-degree relative, then the absolute lifetime risk of NHL is 3.6% in first-degree relatives of an NHL patient. The absolute risk is even lower for specific lymphoma subtypes, which are less common. While the absolute lifetime risk of NHL is not trivial, the relatively low incidence of lymphoma, the modest familial risk, and the lack of a screening test and associated intervention all argue against active clinical surveillance of family members of lymphoma patients based on their family history at this time. The clinical management of familial hematologic cancer syndromes with known germline variants mainly involves AML/MDS and ALL [10].

Such germline variants have not been identified for most lymphomas, and as discussed most inherited risk appears to be due to common variants of weak risk, limiting clinical utility. However, by combing these variants in genetic risk scores, alone or in combination with other risk factors, might improve prediction ability to the level needed in the clinic [367]. While there are currently no validated risk scores for lymphoma, this advance is anticipated as more loci are characterized.

Use of Host (Germline) Genetics in Prognostication

Similarly, evaluation of germline genetics to predict response to therapy, toxicity, and prognosis as part of precision medicine is also largely not yet ready for the clinic. Using germline genomic markers is attractive, since they can be obtained from multiple types of biologic specimens (peripheral blood, buccal cells) and are relatively easily and reliably measured (i.e., genotyped). While some early results suggest germline genetic markers can add important additional prediction beyond clinical and tumor factors or may be used to select treatment type or treatment dose (pharmacogenetics), this remains a future goal in lymphoma. It appears that most SNP markers will only be modestly associated with prognosis (HRs 1.5–2.0), which will require large studies to identify and validate. While these may be individually less useful in a clinical setting, it is hypothesized that by combining multiple markers we can strengthen prediction ability, consistent with a polygenic model of disease pathogenesis. Ultimately, the clinical validity (by assessing sensitivity, specificity, and positive and negative predictive values) will need to be established for genetic markers, along with demonstrated clinical utility to improve the management of lymphoma patients.

Future Directions

In the last few years, large cohorts of most lymphoma and leukemia subtypes have been sequenced, essentially defining the mutation landscape of the diseases. Overall, most diseases are characterized by a considerable genetic heterogeneity and the lack of a pathognomonic genetic event. Furthermore, most of this work has yet to link tumor genomic alterations with treatment response or prognosis. Finally, one of the foremost challenges in the "post-genomic" era is the functional validation of this large number of mutated genes in order to dissect their role in disease pathogenesis.

A major limitation of studying bulk tumors is the inability to resolve cellular states. Currently, we have the ability to monitor global mutation profile in hundreds or even thousands of individual cells in a single experiment, therefore becoming a powerful tool to characterize the tumor architecture and fully characterize the different subclones and their dynamics overtime. Furthermore, single cell data will define the exact combination of driver mutations in each subclone, some of which have targeted therapies in clinical testing or under development and may ultimately translate into novel, individualized treatment approaches. Similar opportunities are originated from the study of circulating tumor cells and cell-free DNAs, which might provide a powerful tool for diagnostic, prognostic and therapeutic approaches in a non-invasive way.

New challenges and research opportunities are also offered by the study of premalignant conditions, especially focusing on the identification of more precise methods to determine which individuals with premalignant precursors will have a clinically significant health condition. Some individuals with premalignant conditions experience relatively rapid progression within 1–2 years while others live for decades without requiring treatment. The prevalence of such conditions will likely increase in the next 2 decades with the aging of the U.S. population. Paradigms in the hematological malignancies are monoclonal B-cell lymphocytosis (MBL) and MGUS, premalignant conditions of CLL and MM, respectively. The feasibility of obtaining sequential samples makes the study of hematological pre-malignancies attractive models to study early carcinogenesis in a longitudinal fashion

At the host level, characterization of genetic susceptibility in lymphoma is rapidly evolving. It is expected that additional common variants will be discovered for the different lymphoma subtypes [304, 368], and perhaps pan-lymphoma loci will also be identified; this genetic architecture should provide new biologic insights to the commonalities and heterogeneity across subtypes. As new lymphoma entities and precursor lesions are defined, evaluation of heritability and genetic susceptibility should be addressed. Additional work needs to occur in other racial and ethnic groups, particularly with contrasting lymphoma incidence rates. It is not yet clear if rare and low-frequency variants will play a major role in lymphoma susceptibility. This will be challenging to address due to phenotype heterogeneity and the need for large sample sizes for these relatively rare entities, and both family and association study designs along with bioinformatics and laboratory-based studies will all need to be integrated to achieve progress [318]. Other genetic mechanisms (e.g., copy number variation), epigenetics, and gene–environment interactions are additional frontiers [317]. Host genetics may also help with prognostication, including immunologic and treatment response, toxicity, and general host response may help to improve prognostic models, individualize cancer treatment (increase efficacy and decrease toxicity), and identify novel treatment targets or approaches.

Finally, integrating somatic and germline genomics is still in its infancy [369], and should provide new insights into lymphoma etiology and pathogenesis, which can be translated to prevention and clinical management, and hopefully provide novel insights into how to prevent and treat this malignancy.

Conclusion

NGS technologies have played an essential role in defining the somatic genomic landscape of lymphomas, with most characterized by a considerable heterogeneity and the lack of a pathognomonic genomic event. From the host perspective, there is strong evidence for familial aggregation across lymphoma subtypes, and this does not appear to be confounded by nongenetic factors, supporting a shared genetic etiology across subtypes. However, a family history of a specific lymphoma subtype is also generally most strongly associated with risk for that specific lymphoma, supporting etiologic heterogeneity. While no major genes have been identified for lymphomas, GWAS have identified over 60 common (MAF > 5%) risk loci, nearly all are associated with specific subtypes. Host genetics has long been hypothesized to influence treatment response, toxicity, and prognosis, but current studies are limited. Ongoing challenges in the "post-genomic" era relate to the functional validation of the large number of mutated genes in the tumor and GWAS loci in the germline, in order to dissect their role in lymphomagenesis. While the use of somatic and host genomics for precision diagnosis, treatment and prevention remains very promising, additional discovery and translational research will be needed before widespread adoption in the clinic.

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39 Epidemiology and Hereditary Aspects of Hodgkin and Non-Hodgkin Lymphomas

Seymour Grufferman

Introduction

The lymphomas have been of unusually great interest to epidemiologists for several reasons. First, both Hodgkin lymphoma and the non-Hodgkin lymphomas have long been suspected of having an infectious etiology and both have been associated with immunosuppression. Second, Hodgkin lymphoma has an unusual pattern of incidence with age, which has suggested that it is at least two different diseases with important underlying environmental determinants. In contrast to Hodgkin lymphoma, the likelihood on clinical and histopathologic grounds that the non-Hodgkin lymphomas are several different disease entities has probably impeded epidemiologic and other research interest in this group of diseases.

The lymphomas have some important common features, however. They are malignancies of the lymphatic system and its precursor cells, which play major roles in the body's defenses against infection and in antibody production. The first isolation of the Epstein–Barr virus (EBV) was from an African Burkitt lymphoma patient. Patients with infectious mononucleosis, caused by EBV, have at least a threefold increased risk of developing Hodgkin lymphoma. Thus, it is not surprising that the lymphomas have frequently been thought to be of viral etiology. Additionally, this group of malignancies is seen with very high frequency in persons with immune deficiencies, ranging from that due to pharmacologic immunosuppression in transplantation to congenital immunodeficiency syndromes or to the acquired immunodeficiency syndrome (AIDS). Interestingly, immunosuppression leads to a remarkably increased risk of non-Hodgkin lymphoma, but only to a slightly increased risk of Hodgkin lymphoma. New evidence has shown that chronic hepatitis-C infection is a cause of non-Hodgkin but not Hodgkin lymphoma [1]. Other infectious agents such as Kaposi sarcoma herpes virus, the human t-lymphotropic virus, and *Helicobacter pylori* have been linked with uncommon subtypes of non-Hodgkin lymphoma but not Hodgkin lymphoma [1]. The lymphomas as a group lead to impaired immune function per se. A clear-cut familial aggregation of the lymphomas is also seen, although family members of a Hodgkin lymphoma patient are likely to have more of that disease but little excess of non-Hodgkin lymphoma and vice versa.

On the basis of known etiologic factors, the two diseases appear to have little in common. EBV is found in up to 50% of all Hodgkin lymphoma tumors, but infrequently in tumors from non-Hodgkin lymphoma patients other than those with endemic Burkitt lymphoma. Environmental and occupational exposures, such as to chemicals, have inconsistently been linked to risk of both diseases. In general, the results of such studies, as presented next, have not been very persuasive. An interesting difference between the two diseases is the much greater frequency with which the non-Hodgkin lymphomas appear as second malignancies when compared with Hodgkin lymphoma. Whereas it appears that heredity plays a large role in the occurrence of both conditions, the patterns of familial aggregation in the two are quite different. Another major difference between the two diseases lies in incidence trends over recent years. The incidence of non-Hodgkin lymphoma worldwide had been increasing at the rate of 3–4% per year, although this increase has tapered off since 1995 in males but not in females (Fig. 39.1). In contrast to this, the incidence of Hodgkin lymphoma has changed little over time and has decreased slightly (Fig. 39.2). Curiously, the increasing trend in non-Hodgkin lymphoma incidence is not evident in China and Japan, countries in which the incidence rates of lymphomas are much lower than in the US. New US data for all ages, races, and both sexes combined (Fig. 39.3) show that the increase in non-Hodgkin lymphoma incidence in the US peaked in about 2007 and has turned down since then. For reasons such as these, the epidemiology and genetics of the two diseases are discussed separately.

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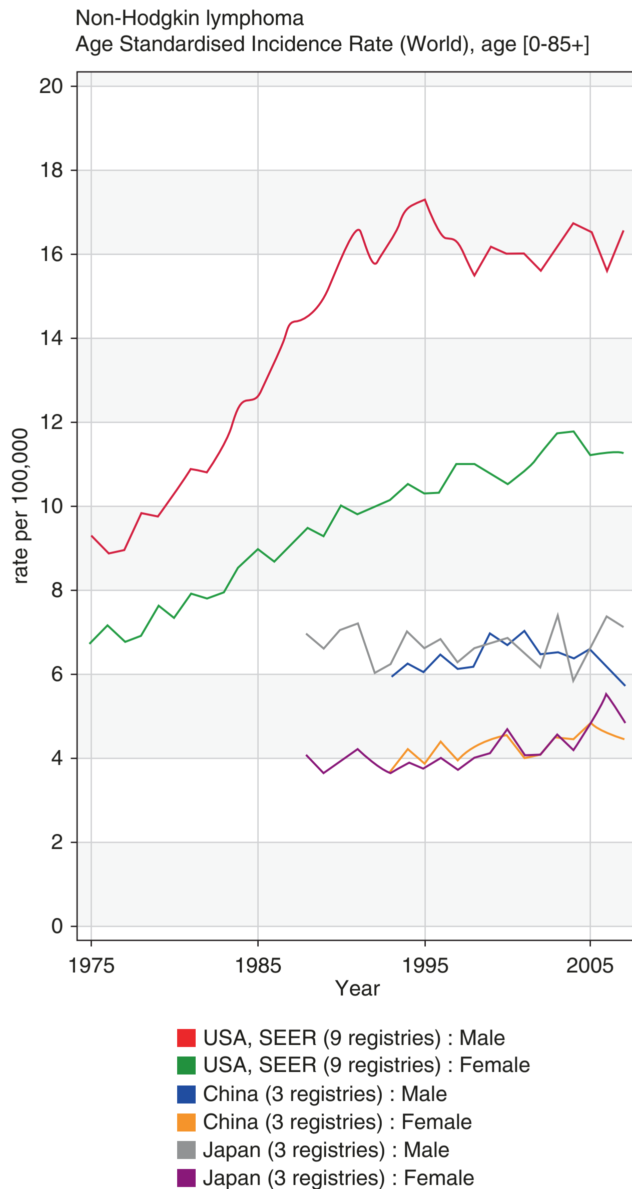

Fig. 39.1 Non-Hodgkin lymphoma. Age Standardized Incidence Rates (World), Age 0–85+. (Modified with permission from Ferley J, Parkin DM, Curado MP, Bray F, Edwards B, Shin HR, Forman D. Cancer Incidence in Five Continents, Volumes I to X: IARC CancerBase No. 9 [Internet]. Lyon, France: International Agency for Research on Cancer; 2017. Available from: http://ci5.iarc.fr/)

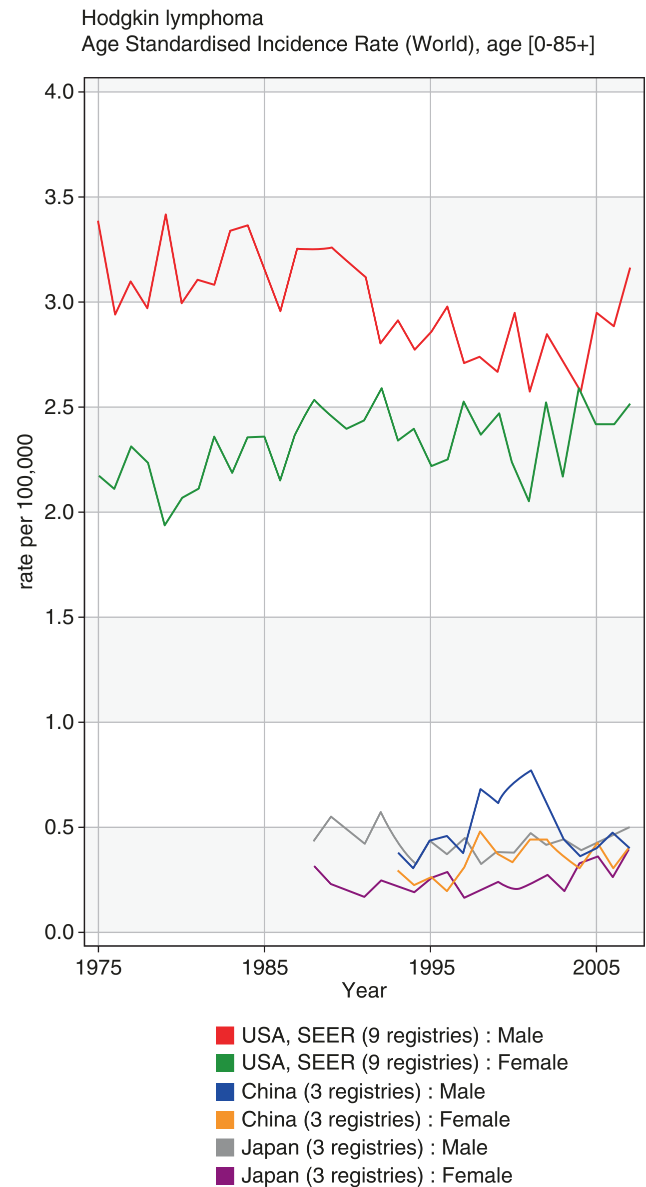

Fig. 39.2 Hodgkin lymphoma. Age Standardized Incidence Rates (World), Age 0–85+. (Modified with permission from Ferley J, Parkin DM, Curado MP, Bray F, Edwards B, Shin HR, Forman D. Cancer Incidence in Five Continents, Volumes I to X: IARC CancerBase No. 9 [Internet]. Lyon, France: International Agency for Research on Cancer; 2017. Available from: http://ci5.iarc.fr/)

Hodgkin Lymphoma

Incidence

Hodgkin lymphoma is a relatively uncommon malignancy. The current overall annual incidence in the US is 2.6/100,000 (3.0/100,000 in males and 2.3 in females) [2]. As can be seen in Table 39.1, incidence rates are higher in whites than in blacks. The overall 5-year survival rate is now up to 88.5% [2]. The improvement in survival is one of modern oncology's greatest triumphs. It should not be forgotten that 50–60 years ago, a diagnosis of Hodgkin lymphoma was a virtual death pronouncement. Unfortunately, progress in effecting greater survival in the elderly Hodgkin lymphoma patient has lagged behind. Table 39.1 shows a diminishing survival with increasing age. Five-year relative survival for patients aged 65 years or older at diagnosis is only 54.2%, possibly because of less aggressive diagnosis, staging, and treatment in this age group [2, 3]. Given the improving general health status of the elderly, this poorer survival should improve in the future.

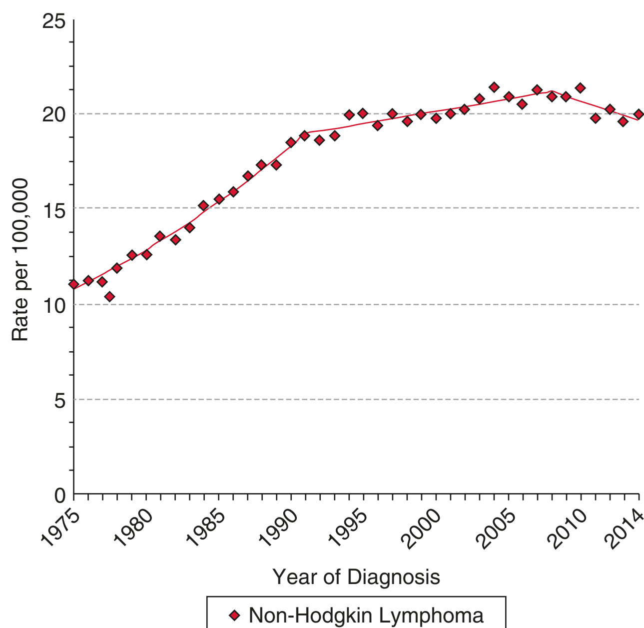

Fig. 39.3 Non-Hodgkin lymphoma,1975–2014. Age-adjusted SEER incidence rates, all ages and races, both sexes. https://seer.cancer.gov/faststats/

Table 39.1 Important statistics on Hodgkin lymphoma

Population	Incidence
US incidence per 100,000 population per year, 2009–2013	
Overall US rate	2.6
Males	3.0
Females	2.3
Whites	2.8
Blacks	2.6
0–14 years	0.6
<65 years	2.4
Males	2.7
Females	2.2
65+ years	3.8
Males	4.8
Females	3.1
Population	Survival (%)
US 5-year relative survival, 2006–2012	
Overall	88.5
Males	87.3
Females	89.8
Whites	89.0
Blacks	85.6
Survival by age at diagnosis	
0–14	97.7
<45 years	94.3
65+ years	54.2

Rates are adjusted to the 2000 US standard population
Based on SEER data, 2009–2013 and 2006–2012

By Age and Sex

Hodgkin lymphoma incidence varies greatly with age. MacMahon, in 1957, first noted a bimodal age–incidence curve with modal peaks in young adults (aged 15–34 years at diagnosis) and old adults (50 years or more) [4]. He postulated that these represented two different diseases, an inflammatory granulomatous infectious disease in young adults and a true neoplasm in the old adults. He noted that the young–adult disease was probably infectious in nature, although of very low infectivity [5]. He also observed that no young–adult incidence peak was seen in Japan and that certain epidemiologic features of childhood Hodgkin lymphoma, such as a striking male excess, suggested that it represented a third type.

The incidence of Hodgkin lymphoma at different ages also varies by sex. A marked excess of males is seen in the childhood form [5]. At the young–adult and old–adult incidence peaks, little or no difference by sex is seen, but a marked male excess is found during middle age (35–59 years). It has been suggested that this gap might be due to a protective effect of childbearing [6], and Glaser [7] presented some data to support this hypothesis. If it is correct, it implies that hormonal factors play a role in the etiology.

International Variation

Observed large international incidence differences have suggested an important etiologic role for environmental factors. The most intriguing observation is that reported in 1971 by Correa and O'Conor [8], who observed that in developed countries such as Sweden and Denmark incidence rates for young–adult onset Hodgkin lymphoma (20–34 years at diagnosis) were quite high and (reciprocally) incidence rates for childhood onset (5–14 years) were quite low. In less developed countries such as Columbia and Nigeria, an inverse pattern was observed—incidence rates were high for childhood disease and reciprocally low for young–adult disease. These observations provided important support for the "poliomyelitis model" or "late-host–response model" of etiology, which is discussed below.

Macfarlane et al. [9] revisited the issue of international variations in incidence. Since the Correa and O'Conor paper was published, diverging incidence trends in childhood (stable rates) and young adults (increasing rates) have been observed [9]. Using incidence rates (1983–1987) from the same source as Correa and O'Conor, Macfarlane et al. [9] found no association between young–adult and childhood incidence. They also found that incidence rates for Latin American countries had shifted toward patterns observed in

more economically developed countries. Thus, the international incidence of the disease is not only different between countries but has changed over time. Such observations suggest that environmental factors play an etiologic role.

Another observation on international variation similarly has not stood the test of time. MacMahon noted in 1966 [5] that no young–adult peak in the age–incidence curve was found in Japan. As seen in Figs. 39.4 and 39.5, the Japanese incidence data from the International Agency for Research on Cancer (1998–2002) now show a definite young–adult incidence peak in males and females [10].

Figures 39.4 and 39.5 depict age–incidence patterns for the United States and two selected Asian countries, Japan and China. These countries were chosen because they have registries that provide stable rates and to demonstrate the overall lower incidence in Asia than in the United States. It is important to note that semi-logarithmic scales are used so that equal distances between curves represent equal proportional differences. In the countries illustrated, male rates are generally higher than those for females, particularly at older ages. Asian incidence rates are several-fold lower than those in the United States. New data plotted on an arithmetic scale (Fig. 39.6) show more clearly that the young adult incidence peaks in Japan and China persist but are of very low magnitude compared to the US. Whether these observations are due to different genetic susceptibility in Asia or to different environmental exposures is unknown. The observation of an emerging young–adult incidence peak in Japan and China coupled with the Westernization of many environmental exposures in these countries suggests that environmental

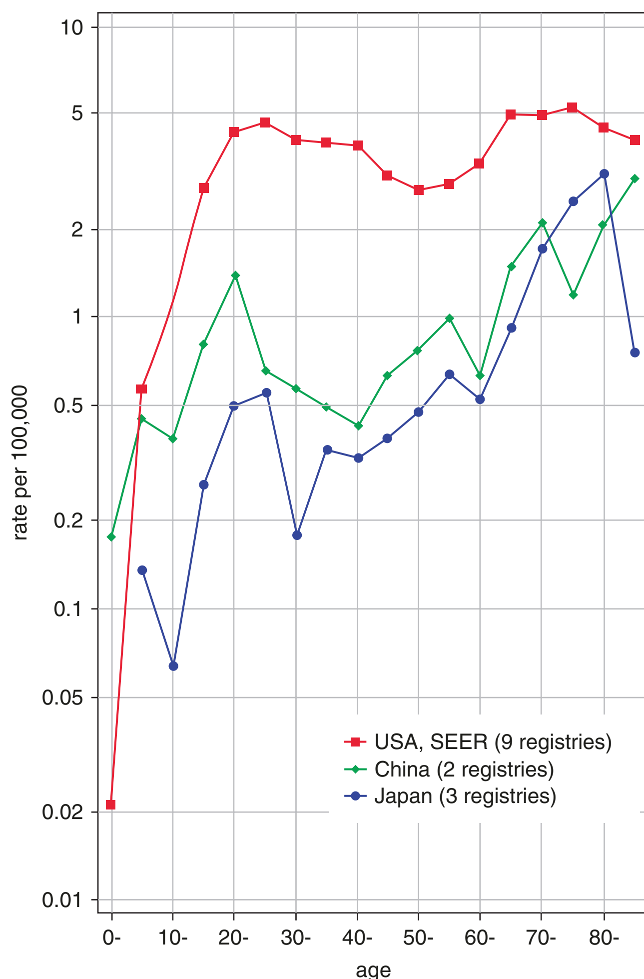

Fig. 39.4 Hodgkin lymphoma, 1998–2002: Male. (Modified with permission from Ferley J, Parkin DM, Curado MP, Bray F, Edwards B, Shin HR, Forman D. Cancer Incidence in Five Continents, Volumes I to IX: IARC CancerBase No. 9 [Internet]. Lyon, France: International Agency for Research on Cancer; 2010. Available from: http://ci5.iarc.fr/)

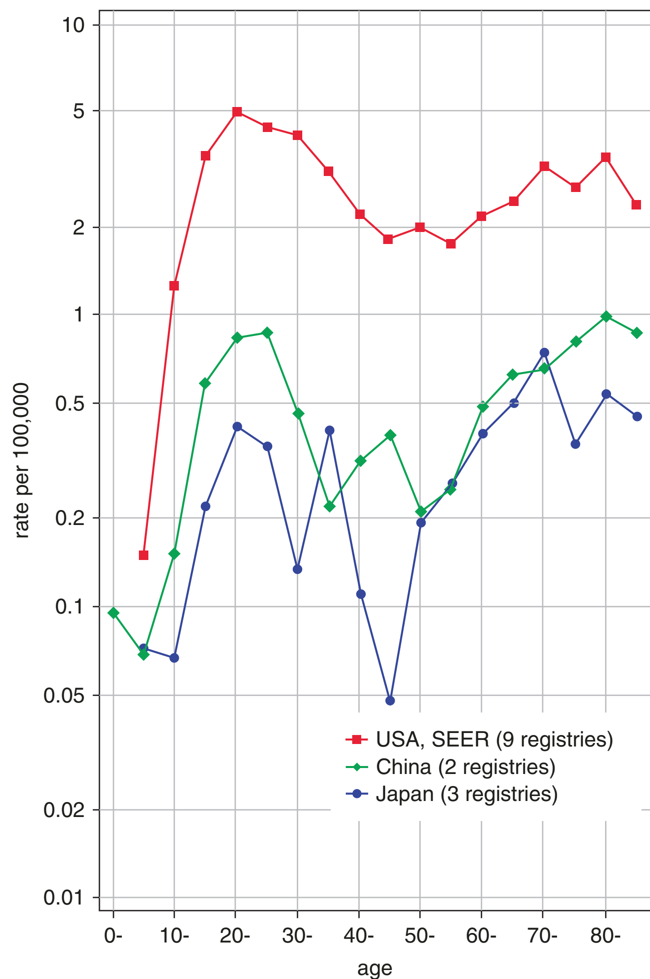

Fig. 39.5 Hodgkin lymphoma, 1998–2002: Female. (Modified with permission from Ferley J, Parkin DM, Curado MP, Bray F, Edwards B, Shin HR, Forman D. Cancer Incidence in Five Continents, Volumes I to IX: IARC CancerBase No. 9 [Internet]. Lyon, France: International Agency for Research on Cancer; 2010. Available from: http://ci5.iarc.fr/)

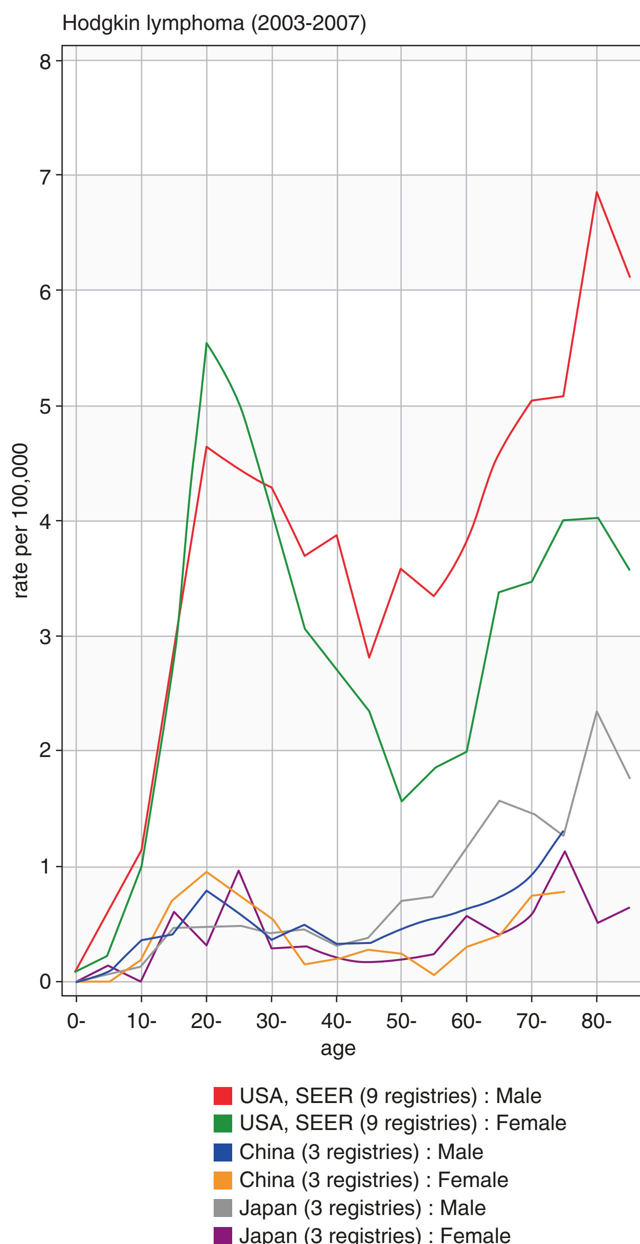

Fig. 39.6 Hodgkin lymphoma, 2003–2007: Age-standardized incidence rates (World), males and females, ages 0–85+

factors may play a greater role than genetic factors at least for young–adult Hodgkin lymphoma.

In summary, large differences are found in the incidence of Hodgkin lymphoma internationally, and they vary by age. Whereas the previously reported reciprocal variation internationally between childhood and young–adult incidence and the virtual absence of young–adult cases in Asian countries are no longer found, there is still very low incidence of the disease in Asia. Observations such as these make a strong case for the importance of environmental factors. They also suggest to some degree that genetic susceptibility plays a role.

Socioeconomic Status

Several intriguing associations between socioeconomic status (SES) and Hodgkin lymphoma risk have been noted. The descriptive epidemiologic findings regarding international variations suggest that less developed countries with their less affluent populations have relatively more childhood and fewer young–adult cases than do developed and more affluent countries. The inverse is seen in more affluent countries. These findings have become less striking with time as observed by MacFarlane et al. [9].

LeShan et al. [11] used data from World War II servicemen to show that men who developed Hodgkin lymphoma generally had higher IQs as measured by the Army General Classification Test. Cohen et al. [12] found that Hodgkin lymphoma patients had more education and were from higher preservice occupational classes compared with World War II servicemen without the disease. Gutensohn and Cole [13] showed that patients with young–adult disease came from smaller families, had fewer playmates, tended to live in more single family dwellings, and came from more affluent and more highly educated families than did controls. Two more recent studies found further results to support the hypothesis of Gutensohn and Cole [14, 15]. Glaser et al. in 2002, reported that Hodgkin lymphoma was weakly associated with a few social class markers but more strongly with combinations of markers in young–adult women. Mack et al. in 2015, reported that among military servicemen serving during 1950–1968, protection from the environment in childhood, but not in adulthood, increased the risk of young–adult Hodgkin lymphoma. Interestingly, they did not confirm the finding of LeShan et al. and Cohen et al. While they found a similar association in servicemen between markers of intelligence and disease risk, these differences were reduced to near unity when more modern adjusted analyses were performed.

Such data support the notion that Hodgkin lymphoma shares epidemiologic features with infectious diseases such as poliomyelitis and infectious mononucleosis. This hypothesis, first proposed by Newell [16], is based on the analogy with paralytic poliomyelitis in the prevaccination era [6]. In less developed countries, exposure to polioviruses occurred early in life, which usually led to a mild or inapparent infection (or both). However, in more affluent countries with better sanitation levels, infection in childhood is much less common. When a nonimmune adult host is infected with the virus, a severe paralytic disease tends to develop rather than the largely inapparent infections of early childhood. This model has also been called the EBV and the late-host–response hypotheses. Young–adult disease would be a rare manifestation of a common exposure.

The first test of this hypothesis was by Paffenbarger et al. [17]. Using data from a follow-up study of college students, they were able to identify alumni at Harvard and the University of Pennsylvania who had developed Hodgkin lymphoma. Data collected at the time of entry to college were used to show that (well in advance of their diagnosis), Hodgkin lymphoma patients reported having had significantly fewer of the common childhood infectious diseases than did matched controls. The later data by Gutensohn and Cole [13] confirm this hypothesis.

In summary, it appears that young–adult-onset patients fit the poliomyelitis model quite well. Unfortunately, extensive data are not available for childhood and old–adult cases to see how these groups may relate to this hypothesis. Nevertheless, the findings strongly suggest that environmental factors play a role, with exposure to viruses best fitting the model. Interestingly, the commonest histologic subtype observed in young–adult disease, nodular sclerosis, has a much lower prevalence of EBV in tumor tissues than does the mixed cellularity subtype [18]. It was the young–adult disease that MacMahon had hypothesized had an infectious etiology [5].

Role of the Epstein–Barr Virus

The possibility that EBV might play a role in Hodgkin lymphoma etiology was suggested by the isolation of the virus from a patient with another lymphoma, African Burkitt lymphoma. Until relatively recently, available laboratory methods could not detect EBV in Hodgkin lymphoma tumor tissues. However, several seroepidemiologic observations had earlier suggested etiologic links between the two conditions [6, 19]. It was well known that patients with Hodgkin lymphoma had higher titers of anti-EBV antibodies than did matched controls, but this was believed to be caused by the immunosuppression of Hodgkin lymphoma per se as well as the immunosuppressive effects of its treatment. In the 1970s, several cohort studies of individuals with serologically confirmed infectious mononucleosis showed that these patients had about a threefold increased risk of eventually developing Hodgkin lymphoma [6, 19]. It could not be discerned from these studies whether the infectious mononucleosis might have been caused by the immunosuppression of underlying (as yet undiagnosed) Hodgkin lymphoma. This concern was dispelled by two nested case–control studies in which serum banks were used to examine antibody status of patients based on blood samples drawn well in advance of any possible diagnosis of Hodgkin lymphoma [20, 21]. These studies showed that tests for anti-EBV antibodies were more apt to be positive and antibody titers higher in patients destined to develop Hodgkin lymphoma than in matched non-Hodgkin lymphoma controls. Thus it would appear that individuals who eventually develop the disease either have different immunologic responses to EBV than the general population or that EBV might be the cause.

Weiss et al. [22] used DNA hybridization to show that EBV DNA could be demonstrated in Hodgkin lymphoma tumor tissues. This innovative work was followed by a large series of studies showing that 30–50% of all adult Hodgkin lymphoma patients will have EBV in their tumors. In situ hybridization studies have since provided direct evidence that the virus is in the malignant cells of Hodgkin lymphoma tumors (Reed–Sternberg cells and their variants) [23].

Additionally, there is molecular evidence that the EBV genome in Hodgkin lymphoma is clonal; it has a restricted latent phenotype of LMP-1 expression and is found in the Reed–Sternberg cells. The consistency of these findings has led the International Agency for Research on Cancer to conclude that the EBV "is a causal factor in the etiology of Hodgkin lymphoma," and they have classified it as a group I human carcinogen [24]. However, not all Hodgkin lymphoma tumors are found to contain EBV, suggesting that there may be etiologic heterogeneity and that not all Hodgkin lymphoma is caused by EBV.

Understanding of the relationship between the EBV and Hodgkin lymphoma is further complicated by the observations of Sleckman et al. [25] that there was no association observed between case's history of infectious mononucleosis and EBV-positivity of their tumors. Further, they also found no association between childhood environmental risk factors and EBV tumor positivity. It must also be recognized that infection by EBV is virtually ubiquitous and 90% or more of most adult populations have serologic evidence of prior infection. Thus, although investigators and the IARC have concluded that EBV is a cause of Hodgkin lymphoma, the causal mechanism is as yet unexplained [18, 24].

In terms of epidemiologic characteristics of EBV-positive cases, 30–50% of all Hodgkin lymphoma cases are EBV-positive, EBV-positivity is greater in less developed countries (>90%) and in HIV-associated cases (>95%), is more common in mixed cellularity than in nodular sclerosis cases, and is more common in the <10 year and >45 year age groups [15].

Genetics

Familial aggregation of Hodgkin lymphoma is well documented, with close relatives having about a threefold increased risk of the disease [26]. Siblings of young–adult cases have a sevenfold increased risk [27]. However, like-sex siblings have a ninefold increase in risk and discordant-sex siblings a fivefold increased risk. No simple

Mendelian genetic mechanism can explain the finding of excesses of male–male and female–female sibling pairs [27]. These excesses suggest that environmental factors might interact with genetic susceptibility. Similar findings of excess sibling sex concordance have also been found in Behçet's disease, multiple sclerosis, and sarcoidosis, a group of diseases with suspected infectious etiology [28]. The underlying assumption is that like-sex siblings have more shared environmental exposures than do unlike-sex siblings. This hypothesis was dealt a setback by a twin study of Mack et al. [29] who found that among twins in which at least one member had young–adult disease, monozygotic twins of patients had a 99-fold increased risk, but dizygotic twins had no increase in risk. These data strongly suggest that the observed familial aggregation is entirely due to shared genetic susceptibility. However, if this were the case, a lower, but still increased, risk should have been observed in dizygotic twins as well. Since this study identified twin pairs by a variety of methods, including advertisements, the results may be due to selection bias and thus need to be confirmed in another, more systematic, study.

Some research suggests that there may be "genetic anticipation" in familial Hodgkin lymphoma. Genetic anticipation is a phenomenon in which each successive generation of a familial disease is diagnosed at earlier ages [30, 31]. This work, if valid, would lend further credence to there being a genetic basis for Hodgkin lymphoma. However, a more recent large, population-based study from Scandinavia found no evidence of anticipation for Hodgkin lymphoma [32]. Thus, there is need for further research on this subject.

Two studies have assessed genetic anticipation in families with a variety of hematologic malignancies that include Hodgkin lymphoma [33, 34]. Both of these studies found significant evidence of anticipation, i.e., younger ages at diagnosis for successive generations. A potential problem with these studies is that they group together several diseases with very different ages of usual onset, so that, for example, greater occurrence of Hodgkin disease in later generations could produce apparent younger ages at diagnosis. Also, follow-up of pedigrees may not be complete enough to include all relatives who go on to develop chronic lymphocytic lymphoma which occurs in the elderly. Nevertheless, the data suggest familial aggregation of several hematologic malignancies and a genetic basis for these diseases.

An interesting study found an apparent genetic parallel between Hodgkin lymphoma and Leri–Weill dyschondrosteosis (LWD) [35]. Based on observations of a sibship in which two sisters had both LWD and Hodgkin lymphoma and of excess sex concordance in Hodgkin lymphoma sibling pairs, the investigators conducted a large study analyzing familial cases. Since LWD has been found to be caused by the homeobox gene SHOX that localizes to the short arm pseudoautosomal region (PAR) of the X and Y chromosomes, they conducted a linkage analysis that suggested that the putative gene for familial Hodgkin lymphoma was in proximity to the site of the SHOX gene in the PAR. Using statistical genetic analyses, they found that PAR- and HLA-linked loci accounted for 29% and 40%, respectively, of the heritability of Hodgkin lymphoma in the US population.

However, the analyses used were questioned by others who used different statistical methods on the same data to find a significant excess of like-sex sibling pairs in Hodgkin lymphoma, unlike the first analysis. They concluded that relying on the analytic methods used, "it is not possible to infer the location or relative importance of a PAR gene for Hodgkin disease" [36]. So this potentially important genetic finding needs further evaluation.

Two studies addressed the question of whether familial Hodgkin lymphoma patients would be more or less apt to have EBV in their tumors. Using similar in situ hybridization techniques, both studies found identical results. In a study of 11 patients from France, 27% had evidence of EBV in their tumors' Reed–Sternberg cells [37]. In a second study of 46 familial cases from the US, 28% of their tumors were EBV positive [38]. Thus, the prevalence of EBV in tumors from familial cases, most of which were of the nodular sclerosis subtype, is similar to that found in nonfamilial patients.

First-degree relatives of Hodgkin lymphoma patients may also be at increased risk of other lymphomas. A very large study by Goldin et al. [39, 40] found a 3.1-fold significantly increased risk of Hodgkin lymphoma in first-degree relatives of cases. However, they also found a 2.1-fold statistically significantly increased risk of chronic lymphocytic leukemia and a 1.3-fold nonsignificantly increased risk of non-Hodgkin lymphoma in first-degree relatives.

Case Clustering

Case clustering in Hodgkin lymphoma has been reported numerous times. Whereas case clustering is characteristic of infectious diseases it can also be a hallmark of common-source exposure to a noninfectious etiologic agent. Vianna et al. [41] noted an unusual cluster of 31 cases that occurred over 23 years and centered around a single graduating class of the Albany (NY) High School. The diagnoses occurred at varying periods after the time of attendance. Twenty-three cases could be directly linked to one another and an additional eight could be linked as case-to-contact-to-case. This was a truly remarkable observation due to the sheer size of the cluster, which is the largest reported so far. Unfortunately, no appropriate statistical methods were used to evaluate its

significance. The Albany study looked at clustering from a different viewpoint, that of shared exposures at a period in time well in advance of diagnosis. This phenomenon has been termed *aggregation of exposures* [42]. It differs from *clustering,* which is defined as increased closeness in time and place of cases at the time the disease is diagnosed. For diseases with suspected long latency periods, aggregation of exposures studies would be much more meaningful than clustering studies.

The Albany results led to another study in high school students on Long Island, NY. Vianna and Polan [43] found that children attending high schools in which a diagnosed Hodgkin lymphoma patient was in attendance had remarkably increased risks of subsequently developing the disease. The estimates of risk ranged from 2.3 to infinity among students exposed to other student cases. This was a remarkable finding that stimulated further research. Unfortunately, an exact replication of the Long Island study conducted in Boston could not confirm the findings [44]. A similar study conducted in Oxford, UK, also could not confirm the Long Island findings [45]. Thus, it appears that no actual confirmed aggregation of exposures related to risk exists in the school setting. More recent studies have used newer biostatistical methods for the study of spatial clustering. Using complicated techniques, Alexander et al. [46]. have shown a possible slight excess of close case pairs over that expected by chance. On balance, there appears to be little or no clustering at the time of diagnosis and no excess of common exposures. Thus, contacts of a patient with Hodgkin lymphoma have no increased risk of developing the disease or other malignancies.

Other Environmental and Occupational Exposures as Risk Factors

Numerous studies have searched for an environmental or occupational cause, with few consistent results. Most of the occupational studies have resulted in variable findings, with most of the positive findings being weak. One of the difficulties in interpreting such studies is that they make multiple comparisons between exposures and diseases. Case–control studies can look at associations with many occupations or exposures. Cohort (follow-up) studies of a particular occupation can evaluate many disease outcomes. Many of the observed disease–occupation associations in such studies are likely to be due to chance, given the large number of associations evaluated. Such chance observations probably underlie many of the reported associations of the lymphomas with occupational exposures. The lack of consistency of findings from one study to another suggests that most reports are indeed chance findings. The strongest suspected occupation is wood-working [6, 47, 48]. As for occupational chemical exposures, many studies have found slight excesses of risk that are not statistically significant [6, 47, 48]. Studies of occupational exposures to herbicides, particularly the phenoxy herbicides, have shown an association, but these results have been inconsistent [49]. On balance, it appears that few, if any, well-confirmed chemical or occupational exposures might cause Hodgkin lymphoma.

A multitude of other potential risk factors for Hodgkin lymphoma ranging from tobacco use to other medical conditions and religion have been studied over the years [5, 6, 13, 19, 48]. Results have been mixed and inconsistent across many studies. The strongest and most consistent risk factors for the disease are listed in Table 39.2.

Table 39.2 Summary of known risk factors for Hodgkin lymphoma

Risk factor	Strength of the association	Consistency of the association
Epstein–Barr virus in Reed–Sternberg cells	Strong	Consistent
History of infectious mononucleosis	Moderate	Consistent
Family history of Hodgkin lymphoma	Moderate to strong	Consistent
Childhood socioeconomic status	Moderate	Consistent
Immunodeficiency syndromes	Weak to moderate	Consistent
Occupational exposures	Weak	Inconsistent

Non-Hodgkin Lymphomas

Incidence

The non-Hodgkin lymphomas are much more common than Hodgkin lymphoma. The recent overall annual incidence in the United States is 19.5 cases/100,000 population (23.7/100,000 in males and 16.1 in females) [2]. As can be seen in Table 39.3, incidence rates are much higher in whites than in blacks and the incidence goes up with age. The incidence rate for males older than 65 years is 112.6/100,000 and for females of the same age group it is 75.4. The 5-year relative survival is 72.6% overall. This survival is much poorer than that of Hodgkin disease. A trend toward poorer survival in the older patient is seen that is not as marked as that observed for survival in Hodgkin lymphoma. As is observed in the latter disease, survival is better in whites than in blacks, but whites have much higher rates of both diseases than do blacks [2].

Table 39.3 Important statistics on non-Hodgkin lymphomas

Population	Incidence
US incidence per 100,000 population per year, 2009–2013	
Overall US rate	19.5
Males	28.7
Females	16.1
Whites	20.4
Blacks	14.6
0–14 years	1.0
<65 years	9.2
Males	10.9
Females	7.5
65+ years	90.9
Males	112.6
Females	76.4
Population	Survival (%)
US 5-year relative survival, 2006–2012	
Overall	72.6
Males	71.7
Females	78.7
Whites	73.6
Blacks	65.2
Survival by age at diagnosis	
0–14 years	90.8
<45 years	81.6
65+ years	62.4

Rates are adjusted to the 2000 US standard population
Based on SEER data, 2009–2013 and 2006–2012

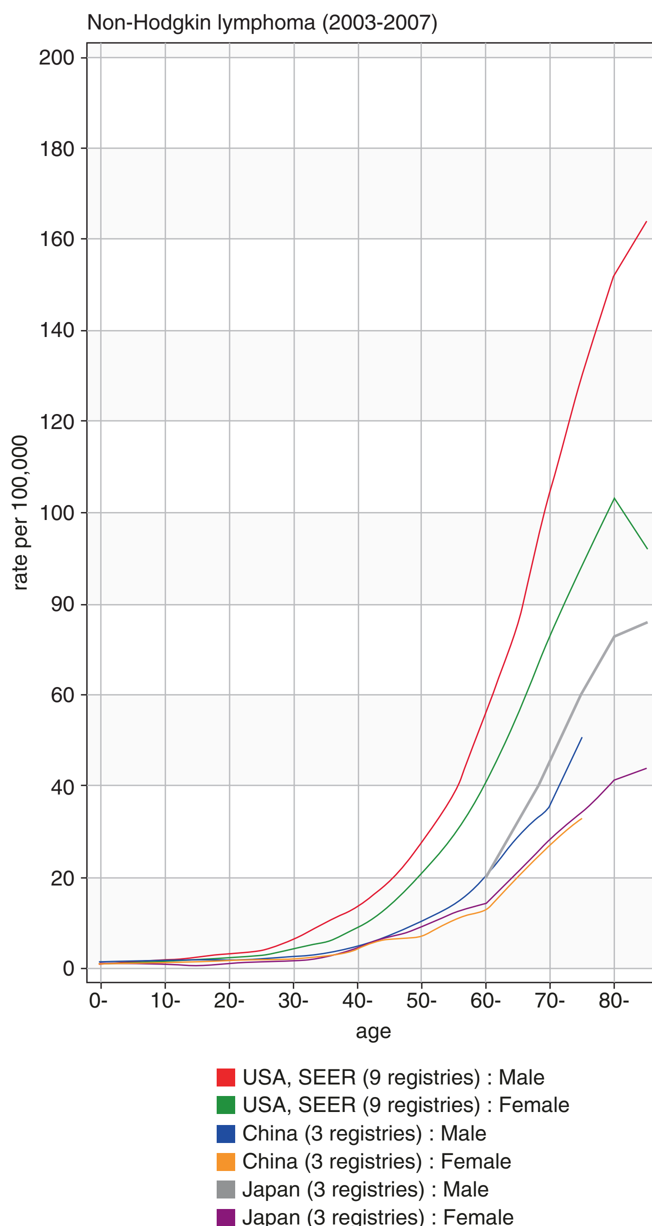

Fig. 39.7 Non-Hodgkin lymphoma, 2003–2007: Male. (Modified with permission from Ferley J, Parkin DM, Curado MP, Bray F, Edwards B, Shin HR, Forman D. Cancer Incidence in Five Continents, Volumes I to IX: IARC CancerBase No. 9 [Internet]. Lyon, France: International Agency for Research on Cancer; 2010. Available from: http://ci5.iarc.fr/)

International Variation

Figure 39.7 depicts non-Hodgkin lymphoma age–incidence patterns for males and females in the United States, China, and Japan [10]. In contrast to the age–incidence pattern for Hodgkin lymphoma that of non-Hodgkin lymphoma shows a unimodal pattern that rises steeply with age after age 30 years. Although the age–incidence plots are of similar shapes in the three countries illustrated, incidence rates consistently are much lower in the two Asian countries than in the US for both Hodgkin and non-Hodgkin lymphomas. In all three countries illustrated, non-Hodgkin lymphoma incidence is higher in males than in females.

Changing Incidence

A great deal of attention has been directed to gradually increasing non-Hodgkin lymphoma incidence rates in the US and developed countries in recent years. Since the early 1970s, US incidence rates have increased at the rate of 3–4% a year, which is more rapid than for all cancers other than melanoma and lung cancer among women [50]. These increasing rates are seen for all ages (except the very young), in whites and blacks, in the United States and internationally, and in both genders. During the 1980s, a further increase was seen in young and middle-aged men related to the AIDS epidemic, but this does not account for all the observed increase in incidence. Increases have been

more marked for extranodal disease, particularly for non-Hodgkin lymphomas arising in the brain, and for high-grade tumors. These observations have stimulated great interest, but no clear explanations have been found. The most recent data from the U.S. SEER program show that this increase has leveled off beginning about 1994 and that it has declined from this peak [2]. Figure 39.3 shows recent SEER data for incidence in the US. These data suggest that non-Hodgkin lymphoma incidence has leveled off and may now be declining. Figure 39.1 shows incidence trends over time for non-Hodgkin lymphoma in the US, China, and Japan. As can be seen, the increasing incidence of non-Hodgkin lymphoma has abated for men in the US since 1995, and only recently for women [10]. The incidence for China and Japan has increased at a slower rate and appears to be declining slightly recently. The comparable incidence data for Hodgkin lymphoma show a relatively stable pattern over the past 30 years (Fig. 39.2). Hodgkin lymphoma incidence rates in the US, China, and Japan have been relatively stable.

It should be kept in mind that the non-Hodgkin lymphomas are a diverse group of diseases. The observed overall increase in incidence may mask significantly more striking increases of individual entities within this overall rubric. Modern lymphoma classification systems such as the WHO classification of tumors of the hematopoietic and lymphoid tissues are based primarily on microscopic morphology and immunophenotypic features of tumors and to a lesser degree on clinical features [51]. Thus, the categories may not correlate with etiologic factors and may not represent distinct etiologic entities. The misclassification of non-Hodgkin lymphomas without regard for causal subgroups could lead to serious misclassification which would mask or artificially produce underestimates of risk in epidemiologic studies. Future classification of the non-Hodgkin lymphomas for epidemiologic research will require close cooperation between epidemiologists and expert pathologists, as well as molecular geneticists, to develop meaningful diagnostic and etiologic entities.

Immunodeficiency

No malignancy, with the possible exception of Kaposi's sarcoma, is more strongly associated with immunodeficiency than is non-Hodgkin lymphoma. The best established cause is impairment of immune function caused by either primary immunodeficiency syndromes, pharmacologic intervention (as used in transplantation), or human immunodeficiency virus (HIV) infection. Observation of this linkage between immunodeficiency and increased lymphoma risk derives originally from studies of cancer occurrence in primary immunodeficiency syndromes. A series of 500 cancers from the Immunodeficiency-Cancer Registry at the University of Minnesota reveals that overall 50.4% of all cancers in these subjects are non-Hodgkin lymphomas [52]. The second most frequent cancers in this registry are leukemias. Hodgkin lymphoma ranks fourth behind adenocarcinoma. The median age at diagnosis for lymphoma cases in this registry is 7.1 years. A 3:1 male/female ratio was seen, which may reflect the contribution of several X-linked disorders as well as the observation of a male excess in tumors of subjects with ataxia-telangiectasia, an autosomal recessive disorder. Most of the non-Hodgkin lymphomas in primary immunodeficiency patients are of B-cell origin. The association between EBV and non-Hodgkin lymphomas occurring in patients with primary immunodeficiencies has not been universal. In this regard, these lymphomas are more similar to those seen in AIDS patients. A high frequency of extranodal non-Hodgkin lymphoma in the primary immunodeficiencies is found, primarily of the central nervous system and gastrointestinal tract.

A second major immunodeficiency risk factor is that of therapeutic immunosuppression, such as in patients undergoing transplantation. The first observations to demonstrate this finding were studies of non-Hodgkin lymphoma risk in renal transplant recipients. It was observed that the disease developed 40–100 times more often than expected and that these tumors had a predilection for the central nervous system, primarily the brain, which was involved in almost half the cases [53, 54]. Most of this excess risk is evident within the first year following transplantation. The risk appears to be related to the intensity of the immunosuppressive therapy, which is reflected in the higher incidence of posttransplant non-Hodgkin lymphoma in heart and pancreas transplant patients, who receive an unusually intensive immunosuppressive regimen [55]. In the overwhelming majority of the lymphomas seen in posttransplant patients, EBV is found in the tumor samples [55]. It is also possible that non-Hodgkin lymphoma occurs with increased frequency in patients without transplantation who received immunosuppressive drugs for conditions such as collagen diseases. Kinlen [56] reported an 11-fold increased risk in patients without grafts who received immunosuppressive drugs for conditions such as rheumatoid arthritis. The increasing use of new pharmacologic and biologic immunosuppressive therapies in a variety of autoimmune diseases, such as psoriasis, rheumatoid arthritis, and Crohn's disease, raises concerns about long-term risks of non-Hodgkin lymphoma in treated patients.

In summary, inherited immunodeficiency syndromes or pharmacologic immunosuppression cause a very striking and large increase in risk. In both instances, the frequencies of both extranodal disease and an aggressive clinical course are high. Whereas posttransplantation non-Hodgkin lymphomas are largely EBV-associated, in a review of tumor

specimens from 13 cases in the University of Minnesota Immunodeficiency Registry only 31% were EBV positive [52]. Immunodeficiency, whether primary, secondary to therapy, or acquired, appears to be the best established and strongest risk factor for non-Hodgkin lymphoma.

AIDS-Related Disease

Non-Hodgkin lymphomas were found to occur remarkably frequently in AIDS patients, an observation that was made quite early in the AIDS epidemic [57]. Further documentation of the AIDS-non-Hodgkin lymphoma association led to the inclusion of non-Hodgkin lymphoma as a diagnostic criterion for AIDS when the criteria were changed in 1985 [58]. Based on epidemiologic surveys, it has been estimated that non-Hodgkin lymphomas occur 60 times more frequently in AIDS patients than do similar lymphomas in the general population [59]. It is estimated that non-Hodgkin lymphoma is the index AIDS diagnosis in about 3% of new AIDS cases, with some evidence suggesting that this frequency may be increasing [60]. AIDS-related non-Hodgkin lymphomas occur in all risk group categories for AIDS (i.e., intravenous drug users, heterosexual contacts of cases, transfusion recipients, and so forth). No differences between risk groups have been reported with regard to clinical manifestations, clinical course, or response to therapy [60]. The patterns of occurrence in HIV patients appear to be changing over time. Earlier in the AIDS epidemic, about 50% of the patients with AIDS-related non-Hodgkin lymphoma had a previous diagnosis of opportunistic infection or Kaposi's sarcoma (or both), and only 15% had no previous AIDS diagnosis. More recently the proportion of such patients with no preceding AIDS-related diagnosis has risen to approximately one-third to one-half.

The impact of HIV infection on incidence has been estimated by modeling techniques. About 2000 AIDS-related non-Hodgkin lymphoma cases occurred in the United States in 1990 [61]. Projections into the future suggest that between 8 and 27% of non-Hodgkin lymphoma cases diagnosed in the United States might be AIDS related. However, it must be remembered that the overall rising incidence of the non-Hodgkin lymphomas in the United States and other countries has clearly preceded the AIDS epidemic and is independent of the contribution of AIDS-related cases.

The clinical presentation of AIDS-related non-Hodgkin lymphoma is similar to that seen in primary, immune deficiency conditions, or other immunosuppressed patients. The non-Hodgkin lymphoma seen in HIV-infected individuals is of high-grade subtypes, with undifferentiated or immunoblastic lymphoma accounting for 60–80% of reported cases of AIDS-related non-Hodgkin lymphoma [61]. Burkitt lymphoma-like tumors account for approximately 20% of AIDS-related non-Hodgkin lymphomas in the United States and seem to occur most frequently in children and young adults between 10 and 19 years of age [62]. Interestingly, most of the Burkitt lymphoma-like AIDS-related non-Hodgkin lymphomas show no evidence of EBV in tumor tissues. Also, most of the AIDS-related Burkitt lymphoma-like lesions are similar to non-endemic Burkitt lymphomas in that they have a low frequency of the characteristic cytogenetic abnormalities seen in the endemic form. However, some AIDS-related Burkitt lymphoma-like tumors in AIDS patients have been found to have the characteristic cytogenetic abnormalities and EBV positivity seen in endemic Burkitt lymphoma.

In general, the AIDS-related non-Hodgkin lymphomas are primarily of B-cell origin, have a rapid progression and aggressive clinical picture with single or multiple tumors, and a predilection for extranodal sites. As in non-AIDS-related non-Hodgkin lymphomas, males are more frequently affected than females. Primary CNS non-Hodgkin lymphoma is a frequent AIDS-related type. Such CNS involvement is rare in non-immunocompromised groups, occurring in approximately 1% of non-Hodgkin lymphomas, but it has been reported to constitute about 20% of those associated with AIDS [59]. Again, it must be pointed out that the increasing incidence of primary CNS lymphoma in the general population appears to be independent of the increase of primary CNS lymphoma in HIV-infected persons [63]. Primary CNS lymphoma may account for up to one-fourth of all HIV-associated non-Hodgkin lymphomas, and involvement of the CNS occurs in 10–25% of patients presenting at other sites. Based on observations such as these, the case definition initially chosen for diagnosis of AIDS was changed to include HIV-positive patients with both high-grade B-cell lymphoma and primary brain lymphomas [56]. It should also be pointed out that the non-Hodgkin lymphomas seen in AIDS may be occult and may only be demonstrated at autopsy [64]. Unfortunately, the precise role of EBV in the AIDS-related non-Hodgkin lymphomas has not been elucidated and may very well differ in various subgroups.

The increasing incidence of AIDS-related non-Hodgkin lymphoma cannot account solely for the increasing incidence of non-Hodgkin lymphoma observed in the United States, and elsewhere. Nevertheless, it is an important contributor to the rise in incidence. It also further suggests that immunodeficiency, whether congenital, acquired, or pharmacologically induced, is a potent risk factor.

The epidemiology of AIDS-related lymphoma has evolved in recent years. The demographic characteristics of these cases have shifted to increasing incidence in Hispanic/Latinos, women, and persons with heterosexually acquired AIDS [65]. The introduction of highly active antiretroviral therapy (HAART) in 1996–1997 has also produced changes in the patterns and incidence of AIDS-related lymphoma.

A large study from France found a marked decrease in incidence of AIDS-related lymphomas in HIV-infected patients from 86.0 per 10,000 person-years in 1993–1994 to 42.9 in 1997–1998 [66]. They also found a greater decrease in primary brain lymphomas from 27.8/10,000 in 1993–1994 to 9.7 in 1997–1998. This may account in part for the recent decline in overall non-Hodgkin lymphoma incidence.

Familial Aggregation

Numerous case reports and series of interesting kindreds have documented the occurrence of more than one non-Hodgkin lymphoma in family members [67]. In comparing aggregation of cancers in Hodgkin lymphoma and non-Hodgkin lymphoma families, several differences are immediately apparent. The first is that for the non-Hodgkin lymphomas far more families are multiply affected (having larger proportions of affected relatives) than are seen in Hodgkin lymphoma. Second, in non-Hodgkin lymphoma, familial aggregation involves relatives with the same disease as well as other lymphoproliferative malignancies, whereas reports of familial aggregation of Hodgkin lymphoma primarily deal with multiple occurrences of the same disease. A third difference relates to reports of multiply affected preadolescent male sibships with extranodal non-Hodgkin lymphomas, primarily of the gastrointestinal tract. Such observations are reported primarily from Mediterranean countries. These may be related to the so-called Mediterranean lymphomas, which are associated with the production of abnormal immunoglobulins. Fourth is the observation that in many multiply affected families primary immunodeficiency syndromes such as ataxia-telangiectasia are also found [67].

Familial aggregation of non-Hodgkin lymphoma appears to be less marked than that observed for Hodgkin lymphoma. Goldin et al., in a large, population-based study from Sweden, found an odds ratio of 1.7 (95% confidence limit = 1.4–2.2) for the occurrence of a non-Hodgkin lymphoma in first-degree relatives of cases. In contrast to this, the same study found an odds ratio of 3.1 (1.8–5.3) for Hodgkin lymphoma in first-degree relatives of Hodgkin lymphoma cases [39, 40]. Interestingly, they also found a slightly increased risk of Hodgkin lymphoma in first-degree relatives of non-Hodgkin lymphoma probands (odds ratio = 1.4, 95% confidence interval = 1.0–2.0). Slight, nonsignificant increases in odds ratios were also found for the occurrence of chronic lymphocytic leukemia (odds ratio = 1.3) and multiple myeloma (1.1) in first-degree relatives of non-Hodgkin lymphoma cases.

Wiernik et al. observed that genetic anticipation occurs in familial non-Hodgkin lymphoma [68]. Genetic anticipation is the phenomenon in which a disease manifests itself at an earlier age or with increased severity in successive generations in a kindred. A significant difference was observed between the ages of onset in the children of cases' generation and the SEER population but not in the parent generation and the SEER population. More recent studies have confirmed this finding, observing genetic anticipation for combinations of Hodgkin and non-Hodgkin lymphomas and for all B-cell lymphomas [33, 34, 69]. If these findings are valid, they would strongly suggest a genetic basis for non-Hodgkin lymphomas and a common genetic basis for both Hodgkin and non-Hodgkin lymphomas. As discussed earlier for Hodgkin lymphoma, studies of anticipation for the combined group of hematologic malignancies could produce biased results due to different ages of onset for the different malignancies included.

A question about the validity of these findings has been raised by a study of anticipation in a large number of Hodgkin and non-Hodgkin lymphoma, chronic lymphocytic leukemia, and multiple myeloma cases from population-based registries in Denmark and Sweden [32]. This study found no anticipation for Hodgkin lymphoma, chronic lymphocytic leukemia, or myeloma. While they found significant anticipation in non-Hodgkin lymphoma families initially, analyses adjusted for the changing incidence rates of non-Hodgkin lymphoma over time led to the disappearance of the statistical difference in the ages of parents and their offspring. They concluded that secular trends in incidence of non-Hodgkin lymphoma could have biased previous observations of anticipation. Thus, there is need for further large, population-based studies to evaluate the validity of the observed genetic anticipation for this group of diseases and resolve the differences in study results.

Skin Cancer and Ultraviolet Exposure

A report by Adami et al. [70] suggests that ultraviolet exposure, as from sunlight, might be one of the factors underlying the increasing incidence of non-Hodgkin lymphoma. This finding fits with an earlier report of an excess of skin cancer in patients with non-Hodgkin lymphoma [71]. Additionally, melanoma and squamous cell cancers of the skin occur excessively, along with non-Hodgkin lymphoma, in renal transplant recipients [53, 54]. These observations, plus the increasing incidence of melanoma, led these investigators to pursue this hypothesis. They reported a fivefold increased risk of squamous cell skin cancer and a twofold increased risk of melanoma in non-Hodgkin lymphoma patients. Conversely, they found a twofold increased risk of the disease in squamous cell skin cancer patients. These findings stimulated much further research. Some subsequent studies found an inverse association between ultraviolet exposure and lymphoma risk [72–75], others a positive association [76], and still others found no association [77, 78].

At present the association remains equivocal, but increasingly appears unlikely to stand the test of time. In many ways, the example of ultraviolet radiation exposure epitomizes the nature of epidemiologic research on the causation of non-Hodgkin lymphoma.

Occupational Exposures

Although many studies of occupations and occupational exposures have been performed, few consistent or strong observations have been reported. One area of major investigation has been farming occupations: at least 21 cohort studies of farmers or occupational surveys of cancer that presented data on non-Hodgkin lymphoma and farming occupations had been done as of 1991 [79]. The risk ratios for the association between this disease and farming ranged from 0.6 to 2.6. Eleven of these 21 studies reported excess risks in farmers, but only three of these were statistically significantly in excess. One of the 21 surveys showed a significantly low relative risk, implying a protective effect. Even more studies of farmers have been done since with similarly inconsistent results [80].

The lesson to be learned from this and other arrays of occupational studies is that many studies have been done of numerous occupations and numerous cancers. Hundreds, if not thousands, of associations have been assessed, and it must be remembered that five of each 100 might be expected to deviate significantly from the null hypothesis just on the basis of chance. Thus, for an assumption of causality to be made for such associations, factors such as the consistency of the findings, the strength of the reported association, the biologic plausibility of the association, and observation of dose–response relationships need to be considered. Using such criteria, it would appear that a slightly increased risk is consistently found in farmers. In general, the magnitude of the increased risk is below twofold and could be due to unidentified confounding factors.

The observation of a possible increased risk in farmers led to studies of chemicals used by these farmers. From these studies have emerged observations that there appears to be an association between pesticide exposure and risk. Several studies have evaluated the potential etiologic role of phenoxy herbicides [81]. Although the results lack consistency, the findings are more consistent than are those from studies of farming occupation and other farming exposures. A dose–response relationship has been suggested in some of the studies as well. At present, no consistent association appears to exist between herbicide use (particularly phenoxy herbicides) and risk of NHL [49, 81]. Alexander et al. reviewed the phenoxy herbicide literature including farming and manufacturing exposures and found associations both positive and negative with the associations centered around the "no association" value of 1.0. [80] Thus, it is likely that there is no meaningful association between occupational exposure to phenoxy herbicides and risk of non-Hodgkin lymphoma. A similar set of results for other occupational pesticide exposures and lymphoma risk was also found in the Alexander et al. review.

Other occupational and environmental factors that have been considered include radiation, zoonotic viruses, exposure to wood, hair dyes, numerous chemicals, electromagnetic fields, tobacco use, and a variety of other exposures [80, 82–85]. In general, none of these exposures has been consistently associated with an increased risk. In summary, very large numbers of investigations of the potential etiologic role of environmental and occupational exposures in the non-Hodgkin lymphomas have been conducted; thus far, these exposures appear to play at most a slight role in the overall causation.

A review of the epidemiologic literature on the non-Hodgkin lymphomas published by Alexander et al. provides the most extensive current review on occupations and occupational exposures and non-Hodgkin lymphoma risks [80]. Their conclusion states, "Review and evaluation of the epidemiologic studies of occupational and environmental factors have identified no specific exposure that is consistently associated with NHL." Their review which was funded by a pesticide manufacturer, but which was peer-reviewed, provides a table on all the published studies of pesticide exposure and non-Hodgkin lymphoma that runs for 5¼ pages. What becomes apparent from review of this table are the numerous studies performed, the generally weak associations found and the tremendous inconsistency of the results. A similar table of occupational exposures and risk of non-Hodgkin lymphoma is presented by Scherr and Mueller in an earlier literature review [85]. Their table runs for 6½ pages and shows similar results—weak associations and lack of consistency of findings. These observations suggest that most of the observed associations reported in the literature are due to chance or bias. Despite a huge amount of epidemiologic research on potential occupational and environmental causes of non-Hodgkin lymphoma, no consistent or strong associations have been found. What this suggests is that no such strong or valid associations exist, or that studies of occupational exposures are subject to misclassification of exposures, or to misclassification of lymphomas as a single etiologic entity. It must be understood that the exposures of farmers, for example, entail a wide range of things ranging from fertilizer and pesticide use to animal infections and to diesel fuel and gasoline. A more serious potential limitation of this area of research may be that non-Hodgkin lymphoma may be composed of a group of similar diseases with different etiologies and studies of the overall group could mask specific exposure associations with component etiologic entities.

A recent, very large pooled analysis of ten international case–control studies from the InterLymph consortium assessed various occupations and risk of non-Hodgkin lymphoma and its subtypes [86]. They confirmed previously reported associations between non-Hodgkin lymphoma and farming occupations: field crop/vegetable farm works OR = 1.26, 95% confidence limit 1.05–1.51, and general farm workers OR = 1.19, 95% CI 1.03–1.37. Like other studies of farmers the ORs were very low and barely statistically significant despite the huge number of cases. Other weak associations were found for women hairdressers, charworkers/cleaners, spray painters, electrical wiremen, and carpenters. Specific lymphoma subtype associations were found for diffuse large B-cell lymphoma (DLBCL) and chronic lymphocytic/small lymphocytic lymphoma in women hairdressers, and for DLBCL and peripheral T-cell lymphoma in textile workers. The broad spectrum of associated occupations suggests that many of these findings are due to chance as might be expected in case–control studies. It was disappointing that few subtype-specific consistent associations were found. Yet again, no strong and consistent occupational risk factors were identified.

Epidemiologic approaches in part have proven unsuccessful to date because of inherent study design limitations. Non-Hodgkin lymphoma is not a common disease and most of the exposures studied as risk factors are relatively uncommon in the general population. The most efficient epidemiologic study design for assessing an uncommon exposure would be cohort studies. In such studies, occupational or other exposure groups can be assembled and followed for occurrence of lymphoma. Since lymphomas are relatively uncommon, particularly in younger individuals, only a few cases might be observed in the cohort leading to statistical instability of the results. Conversely, in a case–control study where relatively large numbers of cases can be assembled, any single environmental exposure would be most uncommon, thereby limiting the stability of the results. Factors such as these are likely explanations for the mixed bag of results obtained thus far and for the great variability of the results.

Burkitt Lymphoma

From an epidemiologist's viewpoint, one of the most interesting non-Hodgkin lymphomas is Burkitt lymphoma. Burkitt lymphoma has perhaps stirred up the greatest initial interest in the possible viral etiology of human lymphomas. Denis Burkitt [87] was an Irish surgeon who, while working in Uganda, first drew attention to a lymphoid malignancy primarily involving the jaw in children. This disease, which he called *African lymphoma,* had distinct clinical, pathologic, and epidemiologic features. Burkitt [88] went on what he termed "tumor safaris" throughout Africa to map the distribution of Burkitt lymphoma. He defined a lymphoma belt extending entirely across Africa, but in a manner corresponding to tropical areas of low altitude. Within his lymphoma belt, he found little Burkitt lymphoma in areas of high altitude [89]. Based on his observations, Burkitt concluded that the disease was defined by rainfall and altitude and corresponded closely to those areas in which malaria was endemic. Later it was found that Burkitt lymphoma appeared to be endemic in New Guinea, particularly in its lowland areas. He did not consider malaria itself as the cause since the disease occurred in countries other than Africa or New Guinea where there is no malaria. This led Burkitt to consider an arthropod-borne virus as the etiologic agent. Burkitt sent specimens of his African lymphoma to outside investigators for study. In 1964 Epstein, Achong, and Barr [90] were able to demonstrate a new herpes virus in cultured Burkitt lymphoma cells from one of Burkitt's patients. It was also observed that when this new virus infected cells it immortalized them in tissue culture.

One of the fascinating early findings in the study of Burkitt lymphoma was that of time–space clustering, a characteristic of many infectious diseases [91]. Several studies showed very clear-cut time–space clustering of Burkitt lymphoma in areas of Uganda. These occurrences appear to suggest epidemic behavior of the disease. However, it must be noted that time–space clustering, while characteristic of many infectious diseases can also be due to other noninfectious shared environmental exposures. Also, based on studies in Uganda, Burkitt lymphoma patients appeared to come from families of lower SES [92].

Based on the observed overlapping of malarious areas and the occurrence of Burkitt lymphoma in Africa, many hypotheses were developed that regarded malaria as an etiologic cofactor. While these notions suffice for endemic Burkitt lymphoma, they could not explain the etiology of the sporadic cases that occur in non-malarious developed countries.

EBV was subsequently shown to occur throughout the world and was identified as the causative agent of infectious mononucleosis by Henle et al. in 1968 [93]. EBV was later found to be associated with many other diseases such as nasopharyngeal carcinoma, sarcoidosis, and lupus erythematosus. It is also almost uniformly present in those lymphomas occurring posttransplantation. African Burkitt lymphoma cases have very much higher EBV antibody titers than do controls, particularly viral capsid antigen (VCA) antibody titers [94]. The high titers of EBV VCA antibodies in Burkitt lymphoma patients were found to antedate the occurrence of the tumor by months to years [95]. With the advent of newer molecular biologic techniques, EBV could be detected in Burkitt lymphoma tumor tissues. About 90% of endemic Burkitt lymphoma patients, but only 10–15% of the non-endemic cases, have been found to have EBV in their tumors [60].

Another characteristic of Burkitt lymphoma is a high frequency of chromosomal translocations that occur at specific breakpoints on chromosomes 8, 14, 2, and 22 [62]. t(8:14) is demonstrated in over 90% of endemic Burkitt lymphoma tumors. The remaining 10% of tumors equally show t(8:2) and t(8:22). These translocations appear to be unrelated to the presence or absence of EBV in the tumor tissues. The less common sporadic form of Burkitt lymphoma occurs in economically developed countries with a worldwide distribution. The sporadic form shows a bimodal age incidence pattern, with peaks in children older than in the endemic form and also in adults. Most of the sporadic Burkitt lymphomas are extranodal, with a high proportion of tumors affecting the gastrointestinal tract or CNS rather than the jaw, as seen in endemic disease. Only about 15% of sporadic Burkitt tumors are EBV-associated [62]. Both types represent clonal proliferations of B-cells and both exhibit the same chromosomal translocations. In AIDS-related Burkitt lymphoma, variability of the tumors exists, with some showing the findings of sporadic and others the findings of endemic Burkitt lymphomas. However, most of the tumors show no evidence of EBV [62].

In summary, Burkitt lymphoma appears to be intertwined with EBV. However, EBV is infrequently found in the sporadic form, which is seen in developed countries. Thus, the model developed to explain the occurrence of Burkitt lymphoma in endemic areas (an interplay of EBV infection, malaria, and some as yet unidentified third factor) cannot explain all cases. Nevertheless, the unusual descriptive epidemiologic features of the disease and the well-documented occurrence of time–space case clustering, plus the apparently major role that EBV plays in most of these cases, suggest that Burkitt lymphoma has a viral etiology. It is now widely accepted that EBV is a cause of Burkitt lymphoma although the causal mechanism is unknown [1, 24].

Infectious Agents

In addition to EBV, several other infectious agents have been evaluated and determined to be causes of some non-Hodgkin lymphomas [96]. The human T-lymphotropic virus-I (HTLV-I) has been found to be a cause of adult T-cell leukemia/lymphoma, an uncommon lymphoma with interesting geographic and epidemiologic patterns of distribution [1, 97]. The human herpes virus-8 (HHV-8), also known as the Kaposi sarcoma virus, has been found to be a cause of primary effusion lymphoma [1, 97, 98]. A bacterial agent, *H. pylori*, has been found to be a cause of mucosal-associated lymphoid tissue (MALT) lymphoma [1, 97, 99]. The hepatitis-C virus (HCV) has been determined to be a cause of all subtypes of non-Hodgkin lymphomas [1, 100].

These findings suggest that infectious agents play an important role in the etiology of some types of lymphoma. These lymphoma types tend to be uncommon, but their diversity suggest that the non-Hodgkin lymphomas are a heterogeneous group of diseases with different causes.

Other Causes

Numerous other potential environmental causes of non-Hodgkin lymphoma have been investigated and again the results of these studies have been weak and inconsistent [80, 84]. Factors ranging from cigarette smoking to radiation exposure to diet and body weight have been studied as risk factors for non-Hodgkin lymphoma with equivocal results. Several autoimmune disorders and medical conditions such as Sjögren's syndrome and type-2 diabetes have also been linked to an increased risk of lymphoma [40, 80, 84]. The reader is referred to the review articles cited here for greater detail on these other possible causes [40, 80, 83–85].

Summary

Both Hodgkin lymphoma and non-Hodgkin lymphoma are interesting diseases that have puzzled epidemiologists due to their many tantalizing epidemiologic findings. Until recently, the non-Hodgkin lymphomas had been gradually increasing in incidence, whereas Hodgkin lymphoma incidence has been either steady or slightly decreasing overall. The increasing incidence of non-Hodgkin lymphoma cannot be fully explained by AIDS-related cases. Both diseases have striking international variation in their occurrence, with some of the highest incidence rates occurring in the United States and other developed countries. Both diseases exhibit familial aggregation with an increased risk to relatives on the order of threefold for Hodgkin lymphoma and two- to threefold for non-Hodgkin lymphoma (Tables 39.2 and 39.4). Both diseases show evidence of genetic anticipation. Hodgkin lymphoma has a bimodal age–incidence pattern, whereas non-Hodgkin lymphoma has a unimodal pattern with its mode occurring in the elderly. The bimodality of Hodgkin lymphoma is constant in developed countries whereas in Asia there appears to be a shift toward bimodality that was previously absent. Whereas SES appears to be directly associated with risk of both diseases, far more data exist on this topic for Hodgkin lymphoma than for non-Hodgkin lymphoma. In fact, the SES findings related to Hodgkin lymphoma make a strong case for the importance of environmental factors in the etiology of the disease. However, for both diseases only a few associations between nonviral environmental exposures and risk have been reported consistently. The most intriguing commonality between the two diseases is their association

Table 39.4 Known risk factors for the non-Hodgkin lymphomas

Risk factor	Strength of the association	Consistency of the association
Immune deficiency		
Primary	Strong	Consistent
Posttransplantation	Very strong	Consistent
Other drug-induced	Strong	
Acquired (AIDS)	Moderate to strong	Consistent
Family history of non-Hodgkin lymphoma	Weak to moderate	Consistent
EBV infection	Moderate	Consistent mostly for Burkitt lymphoma
Other infectious agents (hepatitis-C virus, HTLV-1, HHV-8, *H. pylori*)	Weak to moderate	Consistent, mostly for uncommon lymphomas
Occupational exposures	Weak	Inconsistent

with EBV (Tables 39.2 and 39.4), although different patterns of association are seen. However no other infectious agents appear to be causally related to Hodgkin lymphoma, whereas several agents are associated with some uncommon non-Hodgkin lymphomas. The lymphomas represent some of the few human cancers in which a virus can be identified in tumor specimens with any degree of regularity.

For both diseases, the most likely etiologic mechanism is a complex interplay among genetic susceptibility, immune function, and perhaps viral infection (Tables 39.2 and 39.4). The difficulty in unraveling this complex knot is that abnormal immune function could be genetically determined or virally induced. Which virus may cause these diseases is not known. Although EBV is a sufficient cause in some cases, it is not a necessary cause, as evidenced by the many EBV-negative lymphomas. It appears that HIV is not a direct cause of either disease, rather it is more likely that the disturbed immune function caused by HIV infection leads to non-Hodgkin lymphoma and, less frequently, to Hodgkin lymphoma. On these grounds, both groups of diseases would appear to be fertile territory for uncovering important etiologic mechanisms in human cancer.

An important area for future epidemiologic research on the non-Hodgkin's lymphomas is the search for environmental risk factors for component lymphomas, since the rubric of non-Hodgkin lymphoma used in most previous research may well have encompassed several different diseases with different etiologies. It is likely that previous epidemiologic research has been stalemated by reliance on outdated classifications of these lymphomas. The disappointing results of prior epidemiologic research on non-Hodgkin lymphoma subtypes with weak and inconsistent findings may be a reflection of such misclassification of diagnostic entities. Future research will need to go beyond the established subtypes that have been determined largely by histology and immunophenotyping. Collection of patients' biologic specimens (tumor tissues and blood) will also be necessary. Molecular genetic biomarker approaches and newer statistical methods, such as medical informatics as used in cancer biomarker research, may have to be used. Studies searching for new categorizations of lymphomas will have to be of large size and may require multicenter studies to assure adequate numbers of patients with individual lymphoma subtypes. A feasible approach to mounting such studies would be to utilize cases from clinical trials to conduct case–control studies, as has been done for the relatively rare pediatric cancers [101]. Increasingly, cancer clinical trials are being done on highly specific molecular biologic classifications of subsets of cancers. Clinical trials of molecularly defined subtypes of lymphomas would provide carefully diagnosed cases in relatively large numbers for case–control studies of component lymphomas. Unlike prior case–control studies, definitions of cases can then go beyond simple light microscopy and immunophenotyping disease classifications.

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40 Pathology of Non-Hodgkin and Hodgkin Lymphomas

Mariko Yabe and L. Jeffrey Medeiros

Introduction

Non-Hodgkin lymphomas (NHLs) are neoplasms that arise from lymphocytes of B-cell, T-cell, or NK-cell lineage or rarely from histiocytes. The anatomic location and many of the biologic features of various NHL types can be related to their normal counterparts. Normal B-cells are concentrated in the follicles and medullary cords of lymph nodes and in the follicles of the spleen [1]. The lymphoid follicles represent the proliferative site of the B-cell system. Upon antigen stimulation, secondary germinal centers develop in which rapid cell division of B-cells takes place. The medullary cord region of the lymph node represents the secretory component of the B-cell system. By contrast, T-cells are selectively concentrated in the paracortical regions of lymph nodes and within the periarterial lymphoid sheaths of the spleen. In addition, small numbers of T-cells are found within follicles where they help in the induction of B-cell differentiation (follicular helper T-cells) [2]. Histiocytes are preferentially found in the subcapsular and medullary sinuses of lymph nodes and the cords of Billroth in the splenic red pulp. Histiocytes are also a part of the reticuloendothelial system of the liver and lung. Tissue histiocytes have two broad categories of function. Most histiocytes are phagocytic, whereas a minority of specialized cells is involved in antigen processing and presentation to B- and T-cells [1, 2].

In general, the cell of origin or normal counterpart of NHLs is best understood for B-cell NHLs. For example, follicular lymphoma is known to be derived from B-cells within the germinal center of the lymphoid follicle. By contrast, the cell of origin of T-cell NHLs is much less well understood and there is also likely plasticity in cell differentiation in relation to the microenvironment and antigenic stimulation. Origin from a multipotential stem cell also may be relevant and could explain the occurrence of rare composite lymphomas, i.e., one site involved by two different histologic types of lymphoma, each of which shares the same molecular abnormality.

Physiological characteristics of B-cells, T-cells, NK-cells, and histiocytes are also retained in many NHLs and can be used for pathologic diagnosis. The best example is immunoglobulin (Ig) synthesis by B-cells. Since B-cells express either Igκ or Igλ light chain, but not both, immunophenotypic assessment for Ig light chain is a useful clonal marker because a neoplastic clone is derived from a single precursor cell. By contrast, a polyclonal proliferation of cells, derived from many different precursor cells, will express a mixture of Igκ- and Igλ-positive cells [1, 2]. T-cells, in contrast, do not express the equivalent of Ig light chains in B-cells, making the assessment of clonality more challenging by immunophenotypic methods, although polyclonal versus monoclonal patterns of T-cell receptor beta chain antibody expression can be assessed by flow cytometry [3]. For NK-cells, there are no rearrangements of the immunoglobulin and T-cell receptor genes. Skewed expression of killer cell immunoglobulin-like receptors (KIR) detected by flow cytometry can help in the assessment of clonality of a NK-cell population [4].

Numerous monoclonal antibodies reactive against B- and T-cell and histiocytes-associated antigens are also available and can be used, in part, for assigning a stage of differentiation or likely normal cell counterpart of an NHL. Histiocyte

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subsets include follicular dendritic cells (located within the germinal center), interdigitating dendritic cells (located in the paracortex), fibroblastic reticular cells, and Langerhans cells (located primarily in skin).

Molecular studies have greatly contributed to the classification of NHLs and understanding of their pathogenesis. B- and T-cells normally undergo rearrangement of their antigen receptor genes which can be used as markers of clonality in the assessment of lymphoid neoplasms [5]. Distinctive chromosomal translocations also have been identified in certain types of NHL, many of which are related to mistakes in the physiologic antigen receptor gene rearrangement process or result from mistakes in repair of double-stranded DNA breaks result from mistakes in repair of [5–7].

Hodgkin lymphoma (HL) and NHL have been regarded traditionally as distinct diseases based on their differences in clinical features, response to therapy, pathology, and immunophenotype. The diagnosis of classical Hodgkin lymphoma (CHL) depends on the identification of large neoplastic cells with a distinctive immunophenotype (CD15 +/−, CD30+, PAX5 dim+, CD45-) in an appropriate cellular background of inflammatory cells. Nodular lymphocyte predominant Hodgkin lymphoma (NLPHL) is characterized by a nodular, or a nodular and diffuse proliferation of scattered large neoplastic cells with CD15-, CD20+, CD30-, CD45+ immunophenotype. In recent years, evidence has accumulated that the malignant cell in virtually all cases of HL is of B-cell origin [8], albeit a defective B-cell, and therefore the clinical and biologic overlap between HL and NHL becomes less surprising. This is especially true for NLPHL, and recent studies have shown extensive overlap between NLPHL and T-cell/histiocyte-rich large B-cell lymphoma [9].

Brief History of Classification of Non-Hodgkin Lymphomas

The era of modern classification of NHLs began in 1956 with a classification introduced by Henry Rappaport, who initially collaborated with Edward Gall, a coauthor of an earlier classification with Tracy Mallory [10]. The approach using the Rappaport classification was to first divide lymphomas by pattern, either nodular or diffuse, and then subclassify them further on the basis of cytologic features. Tumors composed of lymphocytes that closely resembled normal lymphocytes were termed well differentiated. Poorly differentiated tumors were composed of relatively small lymphoid cells with irregular nuclear contours that less closely resembled normal lymphocytes. Non-Hodgkin lymphomas composed of large cells were designated as histiocytic on the basis of their resemblance to normal histiocytes. Undifferentiated NHLs were composed of cells of intermediate size that failed to demonstrate cytologic features of either lymphoid or histiocytic origin. Mixed NHLs were composed of a mixture of poorly differentiated lymphocytes and histiocytes.

The Rappaport classification was popular with pathologists and clinicians at the time, but as our understanding of the normal immune system improved, scientific inaccuracies became apparent on the basis of the following observations. First, morphologically identical lymphocytes were found to be functionally heterogeneous. The principal classes of lymphocytes are B- and T-cells, but these are also multiple subpopulations as well as minor subclasses of lymphoid cells. Secondly, small lymphocytes are not an end-stage cell or the final product of a differentiation process. Thus, grading lymphocytes as either well or poorly differentiated does not necessarily relate to differentiation. Lastly, so-called histiocytic lymphoma of the Rappaport classification was usually not of histiocytic origin, but derived from transformed lymphocytes.

In an attempt to address these scientific inaccuracies, new classifications were proposed that attempted to relate lymphoid neoplasms more closely to their counterparts in the normal immune system. By the late 1970s, six lymphoma classifications were in use throughout the world. Of these, in retrospect, the classifications of Lukes and Collins [11] and particularly the Kiel classification [12] were most influential. Nevertheless, the plethora of classifications resulted in confusion and controversy. In an attempt to resolve these differences, the National Cancer Institute funded an international study to test each of the major classifications on 1175 NHLs staged and treated in a relatively consistent manner [13]. On the basis of this study, it was concluded that each of the systems was useful in separating large numbers of patients into subgroups with varying survivals and clinical features. Furthermore, no one scheme appeared to be superior to another. As a consequence of these findings, the investigators involved in the study jointly developed a Working Formulation [13]. In this formulation NHLs were divided into three groups based on clinical outcome: low grade, intermediate grade, and high grade. The authors also emphasized that the Working Formulation was not an alternative classification, but rather a common language that could be used to translate one classification scheme into another. The Working Formulation became

popular, and was principally used in the United States for the next decade. In Europe, the Kiel classification remained most popular, and was updated in 1988 [14]. It should be emphasized that the Working Formulation was based purely on histologic data; immunologic and molecular data were not included, and this was one of the principal reasons why the Kiel classification remained popular in Europe.

The application of newly developed immunophenotypic and molecular methods to the study of hematolymphoid neoplasms made it clear that the Working Formulation (and to a lesser extent the Kiel classification) were inadequate. The results of these studies showed that the categories of the Working Formulation were immunologically and molecularly heterogeneous. Pathologists in the US began to informally modify the Working Formulation by including immunophenotypic and molecular data. For example, the Working Formulation category of "malignant lymphoma, small lymphocytic" had CD5+ and CD5- subsets. Other categories such as "malignant lymphoma, diffuse mixed small and large cell" had B-cell and T-cell subsets. By the early 1990s, virtually all hematopathologists who used the Working Formulation were using a much improved, more complex version that included histologic, immunophenotypic, molecular, and cytogenetic data, but in a non-standardized fashion. In the meantime, most European hematopathologists continued to use the Kiel classification.

The International Lymphoma Study Group proposed a new lymphoma classification that was essentially a summary of the available literature, and was built upon many of the concepts of the Kiel classification. This classification, first published in the journal *Blood* in 1994, is known as the Revised European-American Classification of Lymphoid Neoplasms (REAL) [15]. After a very important validation study of this classification organized by Dennis Weisenburger and James Armitage at the University of Nebraska Medical Center [16], the REAL classification became the basis for a revised classification of lymphoid neoplasms by the World Health Organization (WHO), with the third edition in 2001, fourth edition in 2008, and recently an update of the fourth edition (Tables 40.1, 40.2, and 40.3) [17].

In this chapter, we emphasize the pathology of lymphoid neoplasms, most of which present as "lymphoma" as they are designated in the current WHO classification. Plasma cell neoplasms, myeloid neoplasms, and histiocytic processes are covered in other chapters.

Table 40.1 Revised 4th Edition of WHO classification of B-lymphoid neoplasms

Precursor B-cell neoplasms
B-lymphoblastic leukemia/lymphoma, not otherwise specified
B-lymphoblastic leukemia/lymphoma, with recurrent genetic abnormalities
B-lymphoblastic leukemia/lymphoma with t(9;22)(q34;q11,2); *BCR-ABL1*
B-lymphoblastic leukemia/lymphoma with t(v;11q23.3); *KMT2A* rearranged
B-lymphoblastic leukemia/lymphoma with t(12;21)(p13;q22); *ETV6-RUNX1*
B-lymphoblastic leukemia/lymphoma with hyperdiploidy
B-lymphoblastic leukemia/lymphoma with hypodiploidy
B-lymphoblastic leukemia/lymphoma with t(5;14)(q31.1;q32.3); *IL3-IGH*
B-lymphoblastic leukemia/lymphoma with t(1;19)(q23;p13.3); *TCF3-PBX1*
B-lymphoblastic leukemia/lymphoma, BCR-ABL1-like
B-lymphoblastic leukemia/lymphoma with iAMP21
Mature B-cell neoplasms
Chronic lymphocytic leukemia/small lymphocytic lymphoma
Monoclonal B-cell lymphocytosis
B-cell prolymphocytic leukemia
Splenic marginal zone B-cell lymphoma
Hairy cell leukemia
Splenic B-cell lymphoma/leukemia unclassifiable
Splenic diffuse red pulp small B-cell lymphoma
Hairy cell leukemia-variant
Lymphoplasmacytic lymphoma
Waldenstrom macroglobulinemia
Monoclonal gammopathy of undetermined significance (MGUS), IgM
Mu heavy chain disease
Gamma heavy chain disease
Alpha heavy chain disease
Monoclonal gammopathy of undetermined significance (MGUS), IgG/A
Plasma cell myeloma
Solitary plasmacytoma of bone
Extraosseous plasmacytoma
Monoclonal immunoglobulin deposition diseases
Extranodal marginal zone B-cell lymphoma of mucosa-associated lymphoid tissue (MALT)
Nodal marginal zone lymphoma
Pediatric nodal marginal zone lymphoma
Follicular lymphoma
In situ follicular neoplasia
Duodenal-type follicular lymphoma
Pediatric-type follicular lymphoma
Large B-cell lymphoma with IRF4 rearrangement

(continued)

Table 40.1 (continued)

Primary cutaneous follicle center lymphoma
Mantle cell lymphoma
In situ mantle cell neoplasia
Diffuse large B-cell lymphoma (DLBCL), not otherwise specified
Germinal center B-cell type
Activated B-cell type
T-cell/histiocyte-rich large B-cell lymphoma
Primary DLBCL of the central nervous system
Primary cutaneous DLBCL, leg type
EBV positive DLBCL, NOS
EBV+ Mucocutaneous ulcer
DLBCL associated with chronic inflammation
Lymphomatoid granulomatosis
Primary mediastinal (thymic) large B-cell lymphoma
Intravascular large B-cell lymphoma
ALK positive large B-cell lymphoma
Plasmablastic lymphoma
Primary effusion lymphoma
HHV8 positive DLBCL, NOS
Burkitt lymphoma
Burkitt lymphoma with 11q aberration
High-grade B-cell lymphoma, with *MYC* and *BCL2* and/or *BCL6* rearrangements
High-grade B-cell lymphoma, NOS
B-cell lymphoma, unclassifiable, with features intermediate between DLBCL and classical
Hodgkin lymphoma
Provisional entities are listed in italics.

Table 40.2 Revised 4th Edition of WHO classification of T- and natural killer-cell neoplasms

Precursor T-cell neoplasm
T-lymphoblastic lymphoma/leukemia
Early T-cell precursor lymphoblastic leukemia
Natural killer (NK) cell lymphoblastic leukemia/lymphoma
Mature T-cell and NK-cell neoplasms
T-cell prolymphocytic leukemia
T-cell large granular lymphocytic leukemia
Chronic lymphoproliferative disorder of NK-cells
Aggressive NK-cell leukemia
Systemic EBV+ T-cell lymphoma of childhood
Hydroa vacciniforme-like lymphoproliferative disorder
Adult T-cell leukemia/lymphoma
Extranodal NK/T-cell lymphoma, nasal type
Enteropathy-associated T-cell lymphoma
Monomorphic epitheliotropic intestinal T-cell lymphoma
Indolent T-cell lymphoproliferative disorder of the GI tract
Hepatosplenic T-cell lymphoma
Subcutaneous panniculitis-like T-cell lymphoma
Mycosis fungoides
Sezary syndrome
Primary cutaneous CD30 positive T-cell lymphoproliferative disorders
Lymphomatoid papulosis
Primary cutaneous anaplastic large cell lymphoma
Primary cutaneous gamma-delta T-cell lymphoma
Primary cutaneous CD8 positive aggressive epidermotropic cytotoxic T-cell lymphoma
Primary cutaneous acral CD8 positive T-cell lymphoma
Primary cutaneous CD4 positive small/medium T-cell lymphoproliferative disorder
Peripheral T-cell lymphoma, not otherwise specified
Angioimmunoblastic T-cell lymphoma
Follicular T-cell lymphoma
Nodal peripheral T-cell lymphoma with TFH phenotype
Anaplastic large cell lymphoma, ALK positive
Anaplastic large cell lymphoma, ALK negative
Breast implant-associated anaplastic large cell lymphoma
Provisional entities are listed in italics

Table 40.3 Revised 4th Edition of WHO classification of Hodgkin lymphoma

Nodular lymphocyte-predominant Hodgkin lymphoma
Classical Hodgkin lymphoma
Nodular sclerosis classical Hodgkin lymphoma
Lymphocyte-rich classical Hodgkin lymphoma
Mixed cellularity classical Hodgkin lymphoma
Lymphocyte-depleted classical Hodgkin lymphoma

B-Cell Non-Hodgkin Lymphomas

B-Lymphoblastic Leukemia/Lymphoma, Not Otherwise Specified

B-lymphoblastic leukemia/lymphoma (B-ALL/LBL), not otherwise specified, is the most common type of B-ALL/LBL. Approximately 90% of B-ALL/LBLs present as leukemic neoplasms, better known as acute lymphoblastic leukemia (ALL). By contrast, B-LBL without leukemic involvement at the time of presentation is uncommon and represents no more than 10% of all B-ALL/LBLs [18].

Patients with B-ALL/LBL are most commonly young children, under the age of 6 years, who present with ALL. Extramedullary involvement is frequent, with a particular predilection for the central nervous system (CNS), lymph nodes, spleen, liver, and testis. Only a small subset of patients presents as lymphoma, with extranodal sites of involvement being most common, particularly skin, bone, and soft tissue, although a mediastinal mass is rare [19–21]. Regardless of stage, patients require aggressive chemotherapy regimens [22].

Histologically, the normal architecture of the lymph node is effaced by a diffuse, relatively uniform proliferation of cells

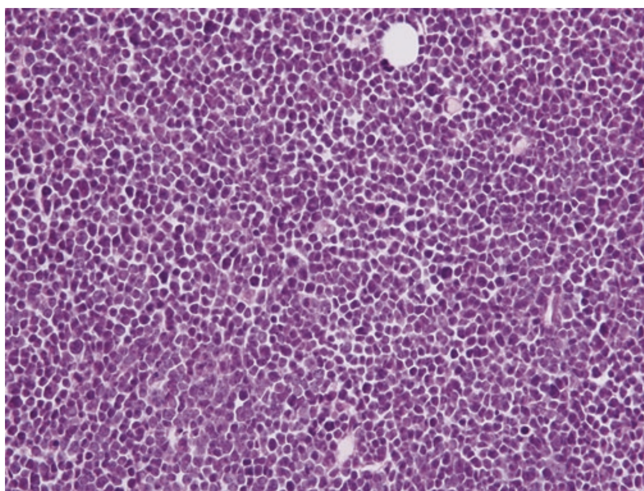

Fig. 40.1 B lymphoblastic leukemia/lymphoma involving adipose tissue. The neoplastic cells are small with blastic chromatin (hematoxylin-eosin, 400×)

that have a tendency to stream out into perinodal tissues [19, 23]. The neoplastic cells have scant cytoplasm, round or convoluted nuclei, and fine (blastic) chromatin (Fig. 40.1). Mitotic figures are numerous. Due to the high growth rate, a starry-sky pattern secondary to individual cell necrosis and scavenging by macrophages can be present in 10–20% of cases.

B-LBLs are neoplasms with immature B-cell immunophenotypes that appear to correspond to "frozen" stages of normal B-cell maturation. Normal B-cells initially express surface CD10 and CD19, followed by CD22, and then CD20. These antigens are detectable in the cell cytoplasm prior to expression on the cell surface. The tumor cells usually do not express Igs on their cell surface [2]. These stages in B-LBL have been referred to as pro-pro B-cell, pro-B-cell, and pre-B-cell, with the latter expressing cytoplasmic IgM in addition to other B-cell antigens. Rarely, surface IgM or Ig light chain (usually not both) can be expressed. An extremely useful marker in the diagnosis of both B-cell LBL is terminal deoxynucleotidyl transferase (TdT), a distinct type of DNA polymerase present normally only in immature lymphocytes [2]. TdT has a physiologic role as it is involved in the process of gene rearrangement, and is thought to add extra nucleotide bases between the variable (V), diversity (D), and joining (J) regions of the immunoglobulin (IG) and T-cell receptor (TCR) genes undergoing rearrangement. TdT is expressed in almost all cases of B-cell LBL.

Similar to surface antigen expression, the antigen receptor genes appear to rearrange sequentially in normal B-cells with a developmental hierarchy [24]. At the earliest stage of B-cell differentiation *IGH* undergoes rearrangement. Subsequently, the Igκ light chain gene rearranges. If neither *IGK* light chain gene allele is functionally rearranged, then the *IGL* light chain gene rearranges. This molecular mechanism allows a B-cell to express only one Ig light chain (the principle of allelic exclusion). The findings in B-LBLs mirror the normal state; all neoplasms carry monoclonal *IGH* rearrangements, but only more mature tumors carry light chain gene rearrangements [24]. Lineage infidelity is common in B-LBLs as T-cell receptor gene rearrangements are common (*TRD* > *TRG* > *TRB*).

B-Lymphoblastic Leukemia/Lymphoma with Recurrent Genetic Abnormalities

A number of nonrandom cytogenetic and molecular findings occur in B-ALL/LBL cases, and some of these abnormalities are sufficiently common that the WHO classification now considers these abnormalities as part of the definition of disease. In sum, there are nine types of B-ALL/LBL associated with specific molecular or cytogenetic abnormalities including two provisional entities (Table 40.1). Histologically, all of these neoplasms are indistinguishable from B-ALL/LBL not otherwise specified (NOS), and there are minor immunophenotypic correlations with specific abnormalities [25]. Details of each entity are discussed in other chapters.

Chronic Lymphocytic Leukemia/Small Lymphocytic Lymphoma

Morphologically and immunophenotypically, the neoplastic cells of chronic lymphocytic leukemia (CLL) are identical to those of nodal-based small lymphocytic lymphoma (SLL), and the two entities are thought to represent different manifestations of the same disease. Many patients who present with nodal-based SLL also have subclinical peripheral blood involvement, and progression to overt CLL occurs frequently. Clinical data also suggest that patients with CLL or SLL can be treated similarly [26]. The designation SLL is now applied to patients who have tissue involvement by a lymphoma that morphologically and immunophenotypically represents CLL/SLL, but with no evidence of leukemia. In the absence of extramedullary tissue involvement, diagnosis of CLL requires $\geq 5 \times 10^9$/L monoclonal lymphocytes with a CLL phenotype in the peripheral blood. The designation of monoclonal B lymphocytosis (MBL) is used for patients who do not fulfill this criterion [27]. Progression to CLL occurs in approximately 1–2% of persons with MBL per year [27].

CLL/SLL represents approximately 12% of all B-cell lymphoid neoplasms. CLL/SLL occurs primarily in middle-aged and older patients with a peak incidence in the sixth to eighth decades. The ratio of male to female cases is approximately 1.5:1. Clinically, CLL/SLL is associated with generalized lymphadenopathy. Although patients frequently have

stage III or IV disease, CLL/SLL patients usually have an indolent clinical course with only vague symptoms such as weakness and anorexia; occasionally they have systemic symptoms [26].

When involved by CLL/SLL, the normal lymph node architecture is usually extensively effaced, but in some cases sinuses are patent. At low power, vaguely nodular, pale areas are usually present that can be small or prominent and represent proliferation centers, also known as pseudo-follicular growth centers or pseudofollicles (Fig. 40.2) [28, 29]. Cytologically, the neoplastic cells are predominantly small round lymphocytes with inconspicuous nucleoli, clumped chromatin, scanty cytoplasm, and infrequent mitoses. However, slightly larger lymphoid cells with irregular nuclear contours (known as prolymphocytes) and large cells with round vesicular nuclei and central nucleoli (known as paraimmunoblasts) are also found. These cells are most numerous in the proliferation centers. In some cases, these proliferation centers can surround residual benign germinal centers mimicking a marginal zone pattern [30].

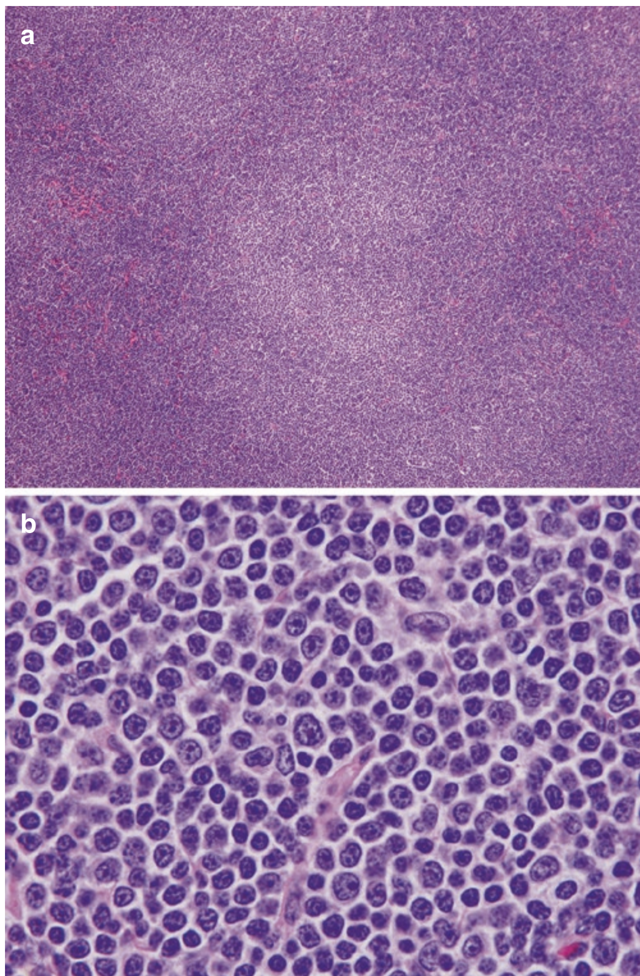

Fig. 40.2 Chronic lymphocytic leukemia/small lymphocytic lymphoma (CLL/SLL) involving lymph node. (**a**) Pale nodules seen at low magnification represent proliferation centers characteristic of CLL/SLL. (**b**) High magnification of proliferation center shows small lymphocytes, prolymphocytes (slightly irregular nuclear contours), and fewer large paraimmunoblasts (with nucleoli). ((**a**) hematoxylin-eosin, 100×; (**b**) hematoxylin-eosin, 400×)

In tissue culture, CLL/SLL cells can be induced to secrete Ig, suggesting that these tumors are composed of cells at a presecretory stage [31]. Furthermore, a small subset of CLL/SLLs exhibit plasmacytoid differentiation. Morphologically, the neoplastic cells in these cases have more abundant cytoplasm and the nucleus may be eccentrically situated. Like plasma cells, these cells contain cytoplasmic Ig that may be secreted producing a monoclonal IgM paraprotein [32]. These patients typically have a low level of monoclonal paraprotein, less than 1.5 g/dL, and these neoplasms behave as do non-plasmacytoid cases of CLL/SLL.

Several studies have suggested that the earliest genetic and epigenetic alterations eventually leading to CLL may occur in pluripotent hematopoietic stem cells (HSCs) [33, 34]. Experiments in mice have shown that HSCs from patients with CLL can engraft efficiently in immunodeficient mice and cause clonal B-cell lymphoproliferations in vivo [33]. These data imply the presence of genetic and epigenetic lesions in HSCs from patients with CLL that skew the potential of HSCs towards the B-cell lineage [33].

Immunophenotypically, CLL/SLL are B-cell tumors that express monotypic Ig light chain (κ > λ), IgM, usually IgD, and pan-B-cell antigens such as CD19, CD20, CD22, CD79A, and PAX5 [35]. The density of Ig and CD20 antigen expression on the surface of CLL/SLL cells is characteristically low corresponding to “dim” immunofluorescence detected by flow cytometry. Bcl-2, CD21, and CD23 are usually positive, and CD79B and FMC7 are usually negative. These neoplasms also express the CD5 antigen, a pan-T-cell antigen that is not expressed on normal B-cells, but is expressed by CLL/SLL. Other T-cell antigens, CD10, Bcl-6, and cyclin D1 are negative. Recently, LEF1 was introduced as a new marker of CLL/SLL. LEF1 is a transcription factor involved in the wnt pathway that is expressed in most cases of CLL/SLL, but rarely in other small B-cell lymphomas [36]. However, it is reported that LEF1 is expressed in ~40% of cases of diffuse large B-cell lymphoma [36].

Chromosomal abnormalities have been shown in CLL/SLL, and are best detected by fluorescence in situ hybridization (FISH) as CLL/SLL cells grow poorly in cell culture [37]. Most FISH panels for CLL/SLL include probes to detect del(13q14), del(11q22) *ATM*, del (6q), del (17p13) *TP53*, and trisomy 12. Del (13q14) is most common (50–60% of patients) and is generally associated with a favorable prognosis, although a subset of patients with deletions involving the retinoblastoma 1 (*RB1*) tumor suppressor gene (−20% of CLL cases) have a less favorable outcome [38, 39]. Deletions of 11q22/*ATM* and (17p13) *TP53* are associated with poorer prognosis. The pathogenetic genes involved

in del(13q14), del(6q), and trisomy 12 are unknown. All together, approximately 75–80% of CLL/SLL cases have abnormalities detected using FISH panels.

A small subset of CLL/SLL cases carries chromosomal translocations further contributing to the molecular heterogeneity of CLL/SLL. Overall, these cases represent less than 10% of all CLL/SLL cases. Translocations involving the *BCL2* oncogene at 18q21 occur in <5% of cases. t(14;18)(q32;q21)/*IGH-BCL2* as well as t(2;18) or t(18;22) translocations involving the *IGK* (chromosome 2p13) or the *IGL* (chromosome 22q11) light chain genes occur. The *BCL3* oncogene, involved in t(14;19)(q32;q13), has been identified in a small subset of CLL/SLL, and correlates with a poorer prognosis and the presence of trisomy 12 [40]. The t(2;11) involving *BCL-11a* occurs in 1–2% of cases.

Genetic analysis of cases of CLL/SLL has shown that this disease has a number of molecular subsets. The variable region genes of the Ig heavy and light chain genes may be mutated (>2% change in germline sequence) or unmutated. Mutated cases of CLL/SLL are clinically indolent and patients have much longer median survival than patients with unmutated CLL/SLL [41, 42]. This may be attributable to defective B-cell receptor signaling in mutated CLL/SLL. As mutational analysis requires sequencing, a relatively labor-intensive procedure, surrogates of Ig mutational status have been sought. ZAP-70 and CD38 have been touted as reasonable surrogates, positive in unmutated cases of CLL/SLL. The correlation, however, is less than perfect.

Recent sequencing studies have shown recurrent mutations in CLL. Mutations in the coding portion of the *NOTCH1* have been reported in up to 10% of patients with CLL at diagnosis, mainly those with CLL of the *IGHV* unmutated subgroup [43–45]. This subgroup shows poorer outcome and increased risk of Richter transformation [46, 47]. Notably, up to 40% of *NOTCH1* mutated CLL patients carry trisomy 12, suggesting functional synergy between these two genetic lesions [48]. Mutations in the splicing factor 3b subunit 1 (*SF3B1*) are observed in up to 10% of the patients, mainly of the IGHV unmutated subgroup, and is associated fludarabine-refractoriness and poorer outcome [43, 49, 50]. Abnormalities of baculoviral IAP repeat containing 3 (*BIRC3)*, gene which regulates the ubiquitin-dependent pathways that modulate NF-κB activation, cause constitutive non-canonical NF-κB activation, and are reported to be associated with poor outcome in CLL/SLL [46]. An additive role of these molecular findings to the traditional FISH-based prognostic model have been proposed, leading to the following classification schema: high-risk CLL/SLL (*TP53* and/or *BIRC3* disrupted); intermediate-risk CLL/SLL (*NOTCH1* and/or *SF3B1* mutated and/or del11q); low-risk CLL/SLL (trisomy 12 or patients with normal karyotype); and very low-risk CLL/SLL (only 13q14 deleted) [51]. Importantly, up to 20% of patients belonging to the low-risk categories by FISH-based analysis were reclassified into higher-risk categories owing to the presence of *NOTCH1*, *SF3B1,* and *TP53* mutations and *BIRC3* disruption [50–52]. This integrated mutational and cytogenetic schema maintains its independent prognostic value during disease progression.

5–10% of cases of CLL/SLL appear to be run in families and there is also evidence for a familial predisposition to CLL/SLL [53, 54]. Several genome-wide association studies have led to the identification of multiple low-risk CLL/SLL susceptibility loci. The genetic basis of familial CLL/SLL, however, is poorly understood.

Approximately 5–10% of CLL/SLL patients develop high-grade lymphoma, also known as Richter syndrome (RS). Molecular findings in CLL/SLL cases that correlate with increased risk of progression to high-grade lymphoma include *NOTCH1* mutations, 8q24/*MYC* translocations, and del(17p13)/*TP53* gene mutations [55, 56]. The most common histologic type of high-grade lymphoma in CLL/SLL patients is diffuse large B-cell lymphoma (DLBCL). About 70–80% of RS-DLBCLs are clonally related to the underlying CLL/SLL and can have unmutated or mutated immunoglobulin heavy chain variable region (IGVH) sequences. Immune deficiency associated with CLL/SLL may be involved in the pathogenesis of clonally unrelated DLBCL. Other less common forms of Richter syndrome include the development of classical Hodgkin lymphoma, plasmablastic lymphoma, and rarely peripheral T-cell lymphoma[57, 58]. Prolymphocytoid transformation of CLL/SLL is also part of the RS spectrum. Approximately half of all cases once designated as B-cell prolymphocytic leukemia are currently better designated as prolymphocytoid transformation of CLL/SLL [59].

Lymphoplasmacytic Lymphoma and Waldenstrom Macroglobulinemia

The terms lymphoplasmacytic lymphoma (LPL) and Waldenstrom macroglobulinemia have evolved in their usage over the past decade, with major changes over the past 15 years. In the current WHO classification, LPL designates a lymphoma composed of small lymphocytes, plasmacytoid lymphocytes, and plasma cells that most often involves the bone marrow, but can involve lymph nodes, spleen, and uncommonly other extranodal sites [60]. A serum IgM paraprotein is very common in LPL. Patients with LPL also can have an IgG or IgA serum paraprotein.

Waldenstrom macroglobulinemia (WM) is considered to be the predominant subset within the LPL category [60–62]. WM is specifically defined as LPL involving the bone marrow that is associated with a serum IgM paraprotein [60]. In other words, WM is a disease of the bone marrow in which a subset of patients has extramedullary disease. Clinically, patients

with WM may present with a variety of symptoms and findings including mucous membrane bleeding, lymphadenopathy, hepatomegaly, peripheral neuropathy, and central nervous system abnormalities [60, 62, 63]. Clinical and laboratory abnormalities that correlate with a poorer prognosis include: age ≥ 65 years, albumin <40 g/L, hemoglobin <11.5 g/dL, platelet count <100 × 10^9/L, beta-2-microglobulin >3 mg/L, and serum monoclonal protein concentration > 7.0 g/dL. These features have been combined into a prognostic index [64]. The serum monoclonal IgM paraprotein concentration is highly variable, with most patients having a paraprotein level greater than 1 g/dL. In our experience, approximately 5–10% of patients present with signs and symptoms of a hyperviscosity syndrome [62]. Lymphadenopathy and hepatosplenomegaly occur in approximately 20% of patients with WM. Leukemic involvement is usually absent, and when present the leukocyte count is usually normal. Lymphadenopathy in WM patients is generalized, but is usually modest.

Histologically, lymph nodes involved by LPL/WM retain their general architecture. The pattern of LPL is diffuse and the cell population is predominantly small lymphocytes, with lesser plasmacytoid lymphocytes, and plasma cells. Intranuclear pseudoinclusions (Dutcher bodies) are common. The neoplastic cells respect sinuses and tend to home to the medullary cord regions [60, 62]. The capsule is extensively infiltrated, and perinodal adipose tissue is involved. It remains challenging to distinguish LPL/WM from nodal marginal zone B-cell lymphoma, particularly in the absence of bone marrow involvement and a serum paraprotein. Histologic evidence of other types of lymphoma that can be associated with a serum paraprotein, for example, proliferations centers as seen in CLL/SLL and monocytoid differentiation as seen in marginal zone B-cell lymphoma, are absent.

The bone marrow is usually replaced by WM in an interstitial or diffuse pattern, but nodular and rarely paratrabecular patterns of involvement occur. Amyloid deposition in the bone marrow or in extranodal sites occurs in small subset of WM patients.

The immunophenotype of LPL is B-cell, with the lymphocytic component expressing surface Ig and B-cell antigens and the plasmacytic component expressing CD19 and cytoplasmic Ig and plasma cell-associated antigens including CD38 and CD138. The Ig heavy chain is usually IgM, but LPL cells can express IgG or IgA. Bcl-2 is positive. The neoplastic cells are usually negative for CD5, CD10, CD23, CD103, and Bcl-6 [65]. CD20 is often expressed variably (dim to moderate or partial).

Next-generation sequencing studies have identified somatic *MYD88* mutations in up to 90% of cases [66, 67]. Although not specific for LPL/WM, *MYD88* mutations are rare or absent in other small B-cell tumors that can mimic LPL/WM, including splenic marginal zone lymphoma (SMZL) (~5%), CLL (~3%), and is very rare or absent in IgM multiple myeloma [67, 68]. Although many different *MYD88* mutations exist, the most prevalent is the L265P missense substitution. In particular, L265P is the sole *MYD88* variant observed in LPL/WM.

In addition to *MYD88*, several additional genes and pathways are concurrently mutated in LPL/WM patients. The chemokine receptor *CXCR4* is the second most commonly mutated gene in LPL/WM (up to 30% of cases) [69]. Nearly all patients with *CXCR4* mutations also carry the *MYD88* L265P mutation. Biologically, of *CXCR4* mutant LPL/WM cells have enhanced signaling downstream of CXCR4 (including BTK activation), as well as increased cell migration, adhesion, survival, and resistance to BTK or PI3Kδ inhibition.

The clinical course of patients with LPL/WM is indolent with median survival ranging from 5 to 10 years in various studies [60, 62]. Approximately 5–10% of patients can develop DLBCL, and, less commonly, LPL/WM patients can develop classical Hodgkin lymphoma. The presence of *CXCR4* mutations negatively affects responses to ibrutinib (BTK inhibitor) treatment in LPL/WM patients.

Follicular Lymphoma

Follicular lymphoma (FL) is a neoplasm of germinal center B-cells that usually exhibits a follicular pattern. Follicular lymphoma is a very common type of NHL in the United States [70]. The median patient age was 59 years in one study, with an age range from 23 to 90 years. Whites are affected more often than blacks. Unlike most other NHLs, women are affected equally or slightly more often than men [70]. Most patients have clinical stage III or IV disease at the time of diagnosis. Involvement of the lymph nodes, spleen, and liver is common. Bone marrow involvement occurs in approximately one-half of patients [70]. A small subset of patients with FL develop clinical evidence of leukemia. The characteristic cell in the peripheral blood has a deeply clefted nucleus and has been referred to as a buttock cell. Leukemic involvement only appears to influence prognosis when the leukocyte count is high. Subclinical involvement of peripheral blood by FL is detected commonly when assessed by molecular methods such as PCR. Rarely pediatric patients can develop FL. This patient subgroup has an excellent prognosis [71].

Histologically, the lymph node architecture is partially or completely effaced by neoplastic follicles, with a paucity of interfollicular tissue (Fig. 40.3). A large absolute number of follicles is the most reliable morphologic criterion for FL [72]. Unlike the lymphoid follicles in reactive hyperplasia, the follicles of FL are relatively uniform in size, lack a well-defined lymphoid cuff, and lack polarization. Histiocytes are usually

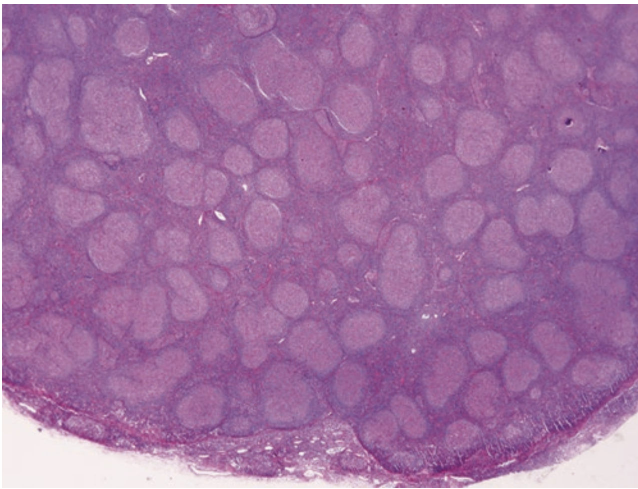

Fig. 40.3 Follicular lymphoma involving lymph node. The neoplasm forms follicles that are spread throughout the lymph node (hematoxylin-eosin, 20×)

less frequent in neoplastic follicles than in reactive follicles. Plasmacytoid differentiation can occur but is rare in FL.

Cytologically, FL cells closely mimic the cell types of normal germinal centers in reactive follicles [70, 72]. Centrocytes have irregular, cleaved nuclear contours and coarse, condensed chromatin. Centrocytes can be small or large (also known as small or large cleaved cells). Centroblasts (also known as large noncleaved cells) are two to three times the diameter of centrocytes with vesicular nuclei and one to three nucleoli. The centroblasts are the proliferating component and therefore the number of mitotic figures correlates with the number of these cells. A small subset of FL, approximately 10%, can exhibit marginal zone (also known as monocytoid) differentiation, manifested by a rim of monocytoid cells located peripheral to the neoplastic follicles. The presence of marginal zone differentiation has been shown to correlate with a poorer prognosis [73].

Grading cases of FL as grades 1, 2, and 3 is based on a count of the centroblasts. In grade 1 FL centroblasts are rare, <5/(high power) 400× microscopic field. Grade 2 tumors have ≥5 centroblasts/400× microscopic field and grade 3 tumors have >15/400× microscopic field. This system also has been modified by subdividing grade 3 FLs into two subsets, 3A and 3B [70, 74]. Grade 3B FL is composed of sheets of centroblasts without centrocytes in the background, and appears to be closely related to de novo DLBCL. The WHO classification suggests that grade 1 and 2 cases of FL can be combined into so-called "grade 1-2" as the clinical behavior of these two patient groups is similar [70]. Others suggest that grade 1, 2, and 3A are similar and can be considered as one group. These opinions will need to be confirmed with follow-up studies.

A component of diffuse pattern is common in biopsy specimens involved by FL, and in some cases the neoplasm can be entirely diffuse without a follicular component. Thus, the presence of neoplastic follicles is highly characteristic of FL, but is not required for diagnosis if the neoplastic cells otherwise have the cytologic, immunophenotypic, and molecular features of FL. Progression from a purely follicular to a follicular and diffuse pattern is common, and the presence of a diffuse component in patients with low-grade FL does not affect survival. A diffuse component, however, usually accompanies an increased number of large cells and therefore a diffuse component is more common in patients with grade 3 FL, particularly grade 3B. When a patient with FL develops a grade 3 tumor that is entirely diffuse, the neoplasm is designated as DLBCL. In biopsy specimens with both follicular and diffuse components, the WHO classification recommends that the components be semi-quantified, especially when the grade of the components is different, for example, follicular lymphoma, grade 1 (50%) with DLBCL (50%).

It is common for an individual patient with FL involving multiple sites to have discordant histologic findings. In this scenario one biopsy site may be involved by low-grade FL and a second biopsy site may be involved by grade 3 FL or DLBCL. This was shown in earlier decades when staging laparotomy was performed for patients with NHL [75]. Discordance is also common when comparing lymph node and bone marrow sites of disease. Lymph nodes may be involved by high-grade FL or DLBCL whereas the bone marrow is involved by grade 1 or 2 FL. In this scenario patient survival is much better than if the bone marrow is involved by grade 3 FL, and therefore bone marrow disease should be graded when possible [76].

Immunophenotypic studies have shown that most FLs express monotypic surface Ig and Igκ is expressed more often than Igλ [77]. Most tumors express IgM, but approximately 25% express IgG or IgA, an expected finding since normal follicular center lymphocytes commonly undergo Ig heavy chain switching following exposure to antigen. IgD is usually negative, since its presence is a feature of naive B cells in the mantle zone. Typically, FLs express Ig and B-cell antigens at high density (bright immunofluorescence). A subset of FLs, mostly grade 3, can be negative for surface Ig.

FLs express pan-B-cell markers, and most are positive for the germinal center B-cell associated antigens CD10, Bcl-6, germinal center B-cell expressed transcript-1 (GCET; also known as centerin), and human germinal center-associated lymphoma (HGAL), and are negative for T-cell antigens including CD5. Within the neoplastic follicles, meshworks of follicular dendritic cells can be appreciated that express CD21, CD23, or other follicular dendritic cell associated antigens. Bcl-2 is expressed in 80–90% of FL; as Bcl-2 is negative in reactive germinal centers, this marker is helpful in differential diagnosis. The centroblasts in FL commonly express activation and proliferation-associated (e.g., Ki-67) antigens. Koster and colleagues have suggested that prolif-

eration rate assessed by Ki-67 immunostaining is prognostically more predictive than grading [78].

The molecular hallmark of FL is the t(14;18)(q32;q21) [79]. Cytogenetic analysis has demonstrated t(14;18) in ~90% of cases. In this abnormality, the *BCL2* oncogene on chromosome 18q21 is juxtaposed with the joining region of the *IGH* gene on chromosome 14q32. The *BCL2* gene is deregulated, by being placed under the influence of *IGH* gene regulatory elements. The presence of t(14;18) alone does not appear sufficient for neoplastic transformation since this translocation has been identified in rare cells in the tonsils, lymph nodes, and blood of normal individuals without clinical evidence of lymphoma [80].

The Bcl-2 protein is a 25-kd molecule that is overexpressed in FLs and protects cells from programmed cell death (apoptosis). The inhibition of apoptosis prolongs cell life, resulting in an expanded compartment of B-cells at increased risk for additional molecular defects, presumably involved in neoplastic transformation. Fluorescence in situ hybridization (FISH) is an excellent way to demonstrate the t(14;18)(q32;q21) as the probes are large and cover most breakpoints [5]. There are at least three important breakpoint clusters in the *BCL2* gene for which PCR have been developed. The two most commonly assessed breakpoint regions are the major (MBR) and minor (MCR) breakpoint cluster regions, involved in 50–60% and 5–10% of cases, respectively [5]. An intermediate cluster region (ICR) also is involved in 10–20% of cases.

Gene expression profiling performed on cases of FL has shown that the host microenvironment has prognostic significance [81]. Molecular signatures corresponding to host T-cell subsets and macrophages are associated with better or worse survival [81, 82]. Another important finding is that gene expression patterns are different in tumors with the t(14;18)(q32;q21) versus tumors without this translocation. Recent advances in next generation sequencing have provided additional insights into the genetic basis of FL. Mutations in chromatin regulator/modifier genes, such as *CREBBP*, *EZH2,* and *KMT2D* (*MLL2*), are common early events, whereas mutations in *EBF1* and regulators of NF-κB signaling (*MYD88* and *TNFAIP3*) are later events and are often present at the time of transformation [83–85]. These genetic alterations can be used in prognostic scoring systems and are potential therapeutic targets [85].

In Situ Follicular Lymphoma

This term describes a specimen involved by FL in which the follicles are of normal size and not easily recognized as FL; however, some of the follicles strongly express CD10 and Bcl-2 (Fig. 40.4) and carry monoclonal *IGH* gene rearrangements and often *IGH-BCL2*/t(14;18)(q32;q21) [86]. Although the term is somewhat "catchy," it may be somewhat misleading as "in situ" in this context does not convey meaning similar to an in situ tumor at other anatomic sites (e.g., cervical carcinoma). A small subset of patients with in situ FL has simultaneous or subsequently develops morphologically obvious FL at other sites. The term in situ *follicular neoplasia* is suggested by the who classification for this lesion.

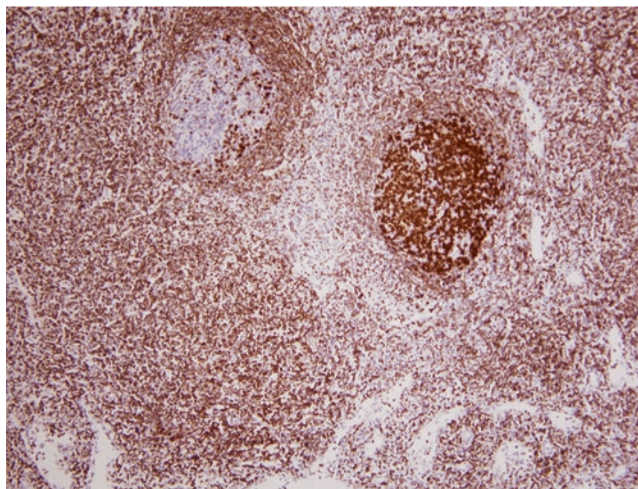

Fig. 40.4 Follicular lymphoma (FL) in situ involving lymph node. In routine histological sections this lymph node looked normal. Bcl-2 immunostaining of the two follicles in this field shows a mostly reactive follicle on the t (Bcl-2 negative) and in situ FL on the right (Bcl-2 strongly positive) (immunohistochemistry with hematoxylin counterstain, 100×)

Extranodal FL

Follicular lymphoma also can arise at extranodal sites. Although the morphologic features of FL at extranodal sites, in large part, resemble neoplasms in lymph nodes, there are important immunophenotypic and molecular differences [70]. Extranodal sites of FL are commonly Bcl-2 negative and lack the t(14;18)(q32;q21). The WHO classification emphasizes the distinctive nature of duodenal-type FL, which has features of a localized low-grade FL, but is distinct from other gastrointestinal tract FL, and has some features resembling in situ follicular FL or MALT lymphoma (see below). Patients with extranodal FL appear to have an excellent outcome, with some patients managed with a watch-and-wait strategy.

Pediatric-Type FL

Pediatric-type FL was recognized specifically in the 2016 update of the WHO classification [17]. These neoplasms preferentially affect boys, who typically present with localized peripheral lymphadenopathy, most often in the cervical region [87]. Pediatric FL typically presents with stage I disease with high histologic grade, expression of CD10 and Bcl-6, and a high proliferation index (Ki67 > 30%), but progression is uncommon and prognosis is excellent [87, 88]. These neoplasms lack *BCL2* or *BCL6* rearrangements, are usually negative for Bcl-2 expression, and often carry *MAP2k1 mutations*.

FL with 1p36 Deletion

A subtype of t(14;18)-negative follicular lymphoma with a predominantly diffuse growth pattern shows deletions in the chro-

mosomal region 1p36 [89]. Patients typically present with painless large lymphadenopathy often in the inguinal area. 75% of patients have localized (stage I or II) disease, and the lymphomas are histologically all low grade. CD23 is often positive. The prognosis is favorable.

Large B-Cell Lymphoma with *IRF4* Rearrangement

This lymphoma typically occurs in Waldeyer ring and/or cervical lymph nodes of children and young adults, and usually presents with low stage [90]. These tumors may have a follicular, follicular and diffuse or pure diffuse growth pattern resembling FL grade 3B or a DLBCL, and strong Irf-4/Mum-1 expression is seen usually with BCL-6 and a high Ki-67 proliferation index. CD10 is expressed in more than half of the cases, and they are most often of germinal center type, particularly based on gene expression profiling studies [90]. Most cases have *IRF4* translocations also involving immunoglobulin gene loci. These tumors uniformly lack *BCL2* rearrangements [90]. Patients with these tumors usually have a favorable prognosis.

Extranodal Marginal Zone B-Cell Lymphoma of Mucosa-Associated Lymphoid Tissue (MALT Lymphoma)

Prior to the advent of immunologic and gene rearrangement techniques, the diagnosis of extranodal low-grade B-cell lymphoma was established infrequently. Extranodal infiltrates composed of small round or slightly irregular lymphoid cells, often admixed with plasma cells, histiocytes, and lymphoid follicles were often classified as *pseudolymphomas*, since clinical studies showed that patients with these lesions pursued an indolent clinical course. Immunophenotypic and gene rearrangement studies have since shown that approximately 60–70% of pseudolymphomas express monotypic Ig light chain and contain immunoglobulin gene rearrangements [91, 92]. Thus, these tumors are now classified as low-grade B-cell lymphomas, and in most cases represent extranodal marginal zone B-cell lymphomas of MALT (MALT lymphoma).

MALT lymphoma was originally described by Isaacson and Wright [93] as a subset of gastrointestinal lymphomas in European patients that resembled immunoproliferative small intestinal disease (IPSID, also known as Mediterranean lymphoma). Subsequently, MALT lymphomas were identified in a variety of extranodal sites including the thyroid gland, thymus, breast, conjunctiva, gallbladder, cervix, larynx, trachea, dura, skin and kidney, as well as other sites [94]. Thus, the term MALT lymphoma, although it continues to be used, is somewhat misleading in the sense that not all MALT lymphomas arise in sites involving mucosal surfaces. Given this wide number of sites involved, the diversity of pathogenesis, and the varied molecular findings (see below), one can now make the argument that these neoplasms should no longer be grouped together as an "entity." However, these tumors do share morphologic and immunophenotypic similarities and are indolent clinically. MALT lymphomas represent approximately 7–8% of all NHL [94].

Patients with MALT lymphoma tend to have localized disease for prolonged intervals before disseminating. This is particularly true for patients with gastric MALT lymphoma whereas patients with non-gastric tumors more often present with stage III or IV disease or disseminate more often [95, 96]. Anatomic sites that are commonly involved by nodal low-grade B-cell NHLs, such as the bone marrow, liver, spleen, and peripheral blood, are infrequently involved by MALT lymphomas. With prolonged follow-up, patients with MALT lymphoma relapse frequently, and relapses often occur in other extranodal sites [94]. In a multivariate analysis at MD Anderson Cancer Center, factors that independently predicted overall survival included elevated serum beta-2 microglobulin level, presence of B symptoms, and male gender [97].

Histologically, four findings are present in most MALT lymphomas: a population of neoplastic small lymphoid (centrocyte-like) cells, occasional large lymphoid cells, reactive lymphoid follicles, and lymphoepithelial lesions when the MALT lymphoma involves a site that normally has glandular epithelium (Fig. 40.5) [94]. The neoplastic small lymphoid cells exhibit a range of cytologic appearances. In some neoplasms these cells closely resemble small lymphocytes, with or without plasmacytoid differentiation. In other neoplasms, the tumor appears biphasic: one component is characterized by small lymphoid cells with minimal cytoplasm, and the other component exhibits extensive plasmacytoid differentiation with many cells resembling mature plasma cells. Plasmacytoid differentiation tends to be more prominent in non-gastric tumors. In other tumors, the neoplastic cells have markedly irregular nuclear contours and resemble small cleaved lymphocytes (centrocyte-like cells). The lymphoma cells may have abundant pale cytoplasm with well-defined cytoplasmic membranes imparting a monocytoid appearance.

In most MALT-lymphomas, occasional large lymphoid cells are also present, which is not surprising since the natural history of a subset of low-grade lymphomas is to accumulate large cells and then progress to a high-grade lymphoma. When the large cells are numerous and form confluent sheets, the neoplasm has evolved to DLBCL. Use of the term *high-grade MALT-lymphoma* is not recommended [94].

Reactive lymphoid follicles are also usually present in MALT lymphomas, usually surrounded by neoplastic small lymphoid cells. These neoplastic cells may accumulate within these follicles (termed *colonization*), and the tumor acquires a nodular low-power appearance [98]. These neoplasms are not truly of follicle center cell origin. When MALT lymphomas involve a site that normally has epithelium (e.g., stomach), the neoplastic cells have a marked tendency to infiltrate epithelium, forming so-called lym-

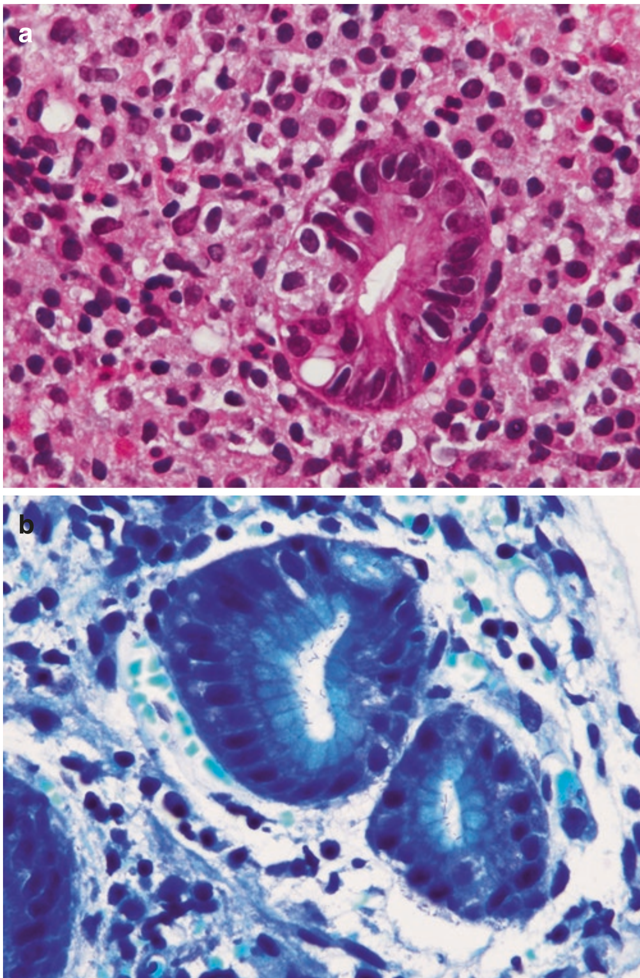

Fig. 40.5 MALT lymphoma involving the stomach. (**a**) Small lymphoma cells infiltrate a gland forming a lymphoepithelial lesion. (**b**) Giemsa stain highlights Helicobacter pylori species on surface of glandular epithelium ((**a**) hematoxylin-eosin, 1000×; (**b**) giemsa, 1000×)

phoepithelial lesions. In these lesions, three or more neoplastic B-cells are found within the epithelium, usually associated with evidence of epithelial damage. In addition to these common histologic findings in MALT lymphomas, there are also important site-specific differences.

In the stomach, normal lymphoid tissue is not present, however, benign MALT is acquired, often in response to *Helicobacter pylori* infection (Fig. 40.5) [94, 99]. Approximately 30% of cases of *H. pylori*-induced chronic gastritis are associated with lymphoid follicles; a smaller subset of cases also develops lymphoepithelial lesions. Conversely, over 75% of gastric MALT lymphomas are associated with *H. pylori* infection. Studies also have shown that benign MALT tissue and MALT lymphomas often regress following antibiotic therapy appropriate for *H. pylori* [100].

Gastric MALT lymphoma is indirectly influenced by *H. pylori* infection through T-cell stimulation, and recent studies have shown that *H. pylori*-triggering chemokines and their receptors, *H. pylori*-associated epigenetic changes, *H. pylori*-regulated miRNA expression, and tumor infiltration by CD4+/CD25+ regulatory T cells all contribute to lymphomagenesis of gastric MALT lymphoma [101–103]. It is also reported that the *H. pylori* protein CagA is incorporated into human B lymphocytes. Intracellular CagA coimmunoprecipitates with SHP-2, suggesting that it stimulates the proliferation of B cells by regulating intracellular signaling pathways, such as the activation of ERK and p38 MAP kinase and upregulation of expression of Bcl-2 [104].

Other associations between infectious agents and MALT lymphoma have been identified. *Chlamydia psittaci* has been associated with up to one-third of MALT lymphomas in the ocular adnexal region, *Borrelia burgdorferi* with a subset of cutaneous MALT lymphomas, and *Campylobacter jejuni* in MALT lymphoma involving the small intestine [105–107]. There are other studies, however, showing a low frequency of infectious organisms in patients with MALT lymphoma. Possibly geographic differences explain these discrepancies.

In the normal adult lung, MALT tissue is also poorly developed and inflammatory conditions usually precede the development of MALT lymphoma. Two inflammatory diseases are frequently associated with lung MALT lymphoma, Sjogren syndrome and lymphoid interstitial pneumonia [94]. Similarly, MALT lymphomas of the salivary gland are usually associated with Sjogren syndrome and Hashimoto thyroiditis frequently precedes MALT lymphoma of the thyroid gland. Patients with Sjogren syndrome have a 44-fold increased risk of developing lymphoma, most of which are MALT lymphomas [94]. Similarly, patients with Hashimoto thyroiditis have a 70-fold increased risk of lymphoma involving the thyroid gland, most of which are MALT lymphomas [108].

Immunophenotypic studies have shown that MALT lymphomas express monotypic Ig light chain, usually IgM, pan-B-cell antigens, and Bcl-2. IRTA1 and MNDA highlight a major subset of MALT lymphomas and appear to be helpful. These tumors typically do not express IgD, CD10, Bcl-6, cyclin D1, or T-cell antigens including CD5. There are no specific immunophenotypic markers for MALT lymphoma.

MALT lymphomas carry monoclonal immunoglobulin rearrangements. In a small study of four patients with multiple sites of disease, VDJ sequence analysis of the *IGH* gene showed that different sites of MALT lymphoma were not clonally related in 3 patients [109]. Approximately 10 chromosomal translocations have been characterized or partially characterized in MALT lymphomas [110]. These translocations, in aggregate, have been shown in approximately 30–40% of MALT lymphomas. These data indicate that MALT lymphoma is highly heterogeneous at the molecular level. Four translocations, to date, are relatively well characterized.

The t(11;18)(q21;q21) has been identified in 20–30% of MALT lymphomas [111, 112]. In this translocation (*BIRC3*) on 11q21 and *MALT1* on chromosome 18q21 are disrupted and recombine to form a novel *BIRC3-MALT1* fusion gene. *BIRC3* belongs to a protein family known as inhibitors of apoptosis proteins (IAP) that are evolutionary conserved and play a role in regulating apoptosis. *MALT1* is a novel gene that encodes a paracaspase that is critical to *BIRC3/API2* is critical to the function of API2-MALT1, which is known to activate NF-κB [113]. This translocation is common in stomach and lung MALT lymphomas.

The t(14;18)(q32;q21) occurs in 10–20% of MALT lymphomas, most often involving the ocular adnexal region, skin, and salivary glands [114]. This translocation juxtaposes *MALT1* at chromosome 18q21 with *IGH* on chromosome 14. This translocation has been implicated in NF-κB activation [113]. The t(3;14)(p14.1;q32) has been reported in up to 10% of MALT lymphomas and most commonly is found in tumors involving the ocular adnexal region, thyroid gland, and skin [115]. t(1;14)(p22;q32) is an uncommon translocation identified in less than 5% of MALT lymphomas that juxtaposes *BCL10* at 1p22 adjacent to *IGH* at 14q32 [116, 117]. The translocation truncates *BCL10* and thus Bcl-10 protein loses its pro-apoptosis function. The t(1;14) occurs most often in MALT lymphomas of the small intestine. *BCL10* mutations also occur outside the context of the t(1;14), in 7–10% of MALT lymphomas [117]. These mutations consist predominantly of deletions or insertions and are predicted to result in truncated proteins.

MALT lymphomas can be divided into translocation-positive and translocation-negative groups. For cases with translocations, the NF-κB pathway appears to be an important common pathway of activation [118]. Translocation-negative MALT lymphomas overexpress genes involved in inflammation and immune response, such as interleukin-8, CD28, and CD86, among others [118]. Other molecular abnormalities have been identified in MALT lymphomas. Trisomy 3 is present in approximately 20% of cases [119]. Trisomies 3 and 18 and del(6q23) occur at a similar frequency among different anatomical sites. Gains at 6p are more common among MALT lymphomas involving the orbital adnexa [120]. Gastric MALT lymphomas have a higher frequency of 8q gains [120]. *MYD88* is deregulated in a small subset of patients with MALT lymphoma (6%), and novel mutation and deletion were reported, in addition to L265P [121, 122].

Alpha Heavy Chain Disease

This disease is also known as immunoproliferative small intestinal disease and as Mediterranean lymphoma [94]. In alpha heavy chain disease the neoplastic cells secrete a form of truncated IgA that cannot bind to Ig light chain, as a result of deletions in the VH or CH1 regions of *IGH*. Morphologically and immunophenotypically alpha heavy chain disease resembles MALT lymphoma, most commonly with extreme plasmacytic differentiation. Patients who live in countries around the Mediterranean Sea or in South Africa are most commonly affected and the disease is associated with poor living conditions. Patients present with diarrhea or malabsorption as a result of disease involving the small intestine and mesenteric lymph nodes. Other gastrointestinal tract sites also can be involved. This disease may respond to antibiotic therapy.

Nodal Marginal Zone Lymphoma

These neoplasms were originally recognized by their cytologic resemblance to reactive monocytoid B-cells, hence the term *monocytoid B-cell lymphoma* was proposed by Sheibani and colleagues [123]. Subsequently, it became more clear that the neoplastic cells differ from reactive monocytoid cells in their location within the lymph node and immunophenotype. These tumors are currently thought to originate in the lymph node marginal zone and are designated as nodal marginal zone B-cell lymphoma (NMZL) in the WHO classification [124]. The designation as nodal (versus extranodal or splenic) in the WHO system is based on arbitrary clinical criteria. A MZL in lymph node is considered as nodal if there is no evidence of extranodal or splenic disease [124]. NMZL represents less than 2% of all NHLs.

Patients with NMZL are usually adults in the sixth or seventh decades with a slight female preponderance in some case series [125, 126]. The clinical course is indolent and B-type symptoms occur in approximately one-third of patients, similar to patients with other systemic low-grade B-cell NHL. Most patients present with peripheral lymphadenopathy, often initially detected as localized lymph nodes in the head and neck region, but widespread lymphadenopathy is also common. Bone marrow involvement is detected at a variable frequency in different studies, ranging from approximately 30 to 60%. Hepatitis C infection is associated with cases of NMZL mostly in Europe [125]. A serum paraprotein, usually at a low level, and composed of IgG, IgA, or IgM, has been reported in up to one-third of patients.

Histologically, lymph nodes involved by NMZL have a pale low power appearance as a result of the abundant cytoplasm of the neoplastic cells. These tumors can show a variety of patterns [124, 127, 128]. A diffuse pattern is most frequent, but NMZLs can colonize follicles imparting a nodular pattern that mimics FL. Uninvolved follicles often contain hyperplastic germinal centers. The cytologic features of NMZL are heterogeneous. The most distinctive cell type is the monocytoid lymphocyte which often predominates in cases of NMZL. The cytoplasm of monocytoid cells is relatively abundant, pale eosinophilic or clear, with well-

delineated cell borders. The tumor cell nuclei are small and cytologically bland. The tumor cell chromatin is relatively clumped and mitotic figures are infrequent. NMZLs also can be composed of centrocytes-like cells or cells with plasmacytoid differentiation. Large cells are also present in variable numbers, but if sheets of large cells are present the neoplasm has transformed to DLBCL. Bone marrow involvement is often observed in NMZL, ranging from 19% to 62% of cases reported in the literature [124, 128].

The diagnosis of nodal marginal zone lymphoma is one of the remaining problem areas in hematopathology, because no established positive markers exist for this lymphoma. Immunophenotypic studies have shown that NMZLs are mature B-cell neoplasms that express monotypic Ig light chain usually IgM, pan-B-cell antigens, CD11c, CD43, and Bcl-2 [124]. IRTA1 and MNDA are positive in a subset of NMZL and are rarely positive in FL [129]. These tumors usually do not express CD10, CD21, CD23, Bcl-6, cyclin D1, and T-cell antigens including CD5. Most cases are negative for IgD but approximately 15% of NMZLs are positive.

The molecular pathogenesis of NMZL is poorly understood. The immunoglobulin (IG) genes are rearranged and the IG variable regions commonly show somatic mutations. Recent gene expression studies have suggested deregulation of the NF-κB pathway [130]. Mutations in PTPRD, NOTCH2, KMT21) KLF2 occur in 20-35% of ases (131).

Multiple studies have investigated the cytogenetic features of NMZL using classical cytogenetics, comparative genomic hybridization, and FISH. No specific alterations have been identified. Gains of chromosome 1q, 2p, 3p, 3q, 6p, and 6q are most frequent, as are losses of 1q and 6q; chromosomes 3, 12, and 18 most often show trisomy, and monosomy is more rarely observed and most frequently involves chromosomes 9, 13, and 14 [121, 131, 132].

Translocations known to occur in MALT lymphoma do not occur in NMZL. Multiple translocations have been sporadically reported in NMZL, but they do not share a common breakpoint region. Some studies have shown translocations involving *BCL6* in a subset of NMZL.

Pediatric Nodal Marginal Zone Lymphoma

Nodal MZLs in children have distinctive clinical and morphological characteristics [17, 133]. There is a striking male predominance (M:F = 20:1), the median age is 16 years old, and 90% of patients present with Stage I lymphadenopathy, usually cervical [133]. Histologically, these tumors are similar to that seen in adults except that there are often features of progressive transformation of germinal center in which the outer border of follicles is disrupted and infiltrated by lymphoma cells [133]. The prognosis of patients with pediatric nodal MZL is excellent.

Splenic Marginal Zone B-Cell Lymphoma

Splenic marginal zone B-cell lymphoma (SMZL) was named by Schmid and colleagues [134] because these neoplasms were thought to arise from splenic marginal zone B-cells, and to be closely related to other marginal zone B-cell lymphomas. The cell of origin of SMZL, however, and its relationship to other marginal zone neoplasms is currently uncertain [135].

Patients with SMZL are adults, usually elderly, and have a characteristic disease distribution: spleen, splenic hilar and other abdominal lymph nodes, and bone marrow. Peripheral blood is often involved and the cells commonly have irregular or villous cytoplasmic projections, hence the older name splenic B-cell lymphoma with villous lymphocytes. Patients present with splenomegaly and laboratory abnormalities, usually anemia or thrombocytopenia, or both [136]. A subset of patients has an associated serum IgM paraprotein and levels can be high [137]. Systemic symptoms are usually absent. Splenomegaly is usually marked, but in a subset of cases the spleen is relatively small and these patients may have early, localized disease. A small subset of SMZL cases harbor hepatitis C virus (HCV), more frequently in southern Europe, and therapy for HCV seems to also affect the control of the tumoral load in these patients [138]. Recently, a high prevalence of SMZL among patients with acquired C1 inhibitor deficiency was reported, although the pathogenesis is not well understood [139].

Grossly, the spleen is usually massive. Histologically, SMZL preferentially involves the marginal zones surrounding lymphoid follicles of the white pulp and, if more extensive, also replaces lymphoid follicles and extends into red pulp [135, 140]. Cytologically, the neoplastic cells have relatively abundant clear cytoplasm and central round, bland nuclei. Plasmacytoid differentiation can be prominent. Mitotic figures are rare and occasional large lymphoid cells are present. Transformation to DLBCL can occur, detected as sheets of large B-cells, and often correlates with a more aggressive clinical course. Almost without exception, SMZL cases have bone marrow involvement. In the bone marrow, neoplasm often shows intrasinusoidal infiltration, and reactive germinal centers also can be present [140].

Immunophenotypic studies have shown that SMZLs are mature B-cell neoplasms that express monotypic Ig light chain, IgM, usually IgD, pan-B-cell antigens, CD11c and Bcl-2. SMZL cells are negative for pan-T-cell antigens, CD10, CD21, CD25, and CD103, and Bcl-6 [135]. Most cases of SMZL are negative for CD5 and CD23, but approximately 20% of cases can be positive for one or both markers. Patients with CD5+ SMZL often have leukemic involvement with high lymphocyte counts raising the differential diagnosis with CLL/SLL [141].

Conventional cytogenetic analysis has shown an abnormal karyotype in up to 75% of cases [135, 142]. Common abnormalities in SMZLs include gains of chromosome 3, more specifically 3q, gains of 12q, and deletions of 6q and 7q. The latter deletions are most often 7q31–32, present in up to 40% of cases. SMZLs contain IG gene rearrangements. The IG variable region genes are commonly mutated [143]. Stereotypes of the IG genes has been shown in approximately 10–30% of cases and are unrelated with the presence of HCV, suggesting a role for antigen drive involved in the pathogenesis of this subset [144]. No well-characterized chromosomal translocations occur in cases of SMZL.

Gene expression profiling of cases of SMZL has shown upregulation of genes involved in intracellular signaling (e.g., *AKT1*) and transcription as well as genes attributable to normal splenic tissue [145]. *MYD88* L265P was detected in up to 15% SMZL cases, and *NOTCH2* mutation has been found in up to 25% of SMZL [146, 147]. A recent study of whole exome sequencing identified novel recurrent inactivating mutations in Kruppel-like factor 2 (*KLF2*) in 42% of SMZLs, but rarely in other B-cell lymphomas [148]. Most *KLF2* mutations were frameshift indels or nonsense changes, with missense mutations clustered in the C-terminal zinc finger domains that suppress NF-κB activation [148]. IGHV1–2 rearrangement and 7q deletion are more common in SMZL with *KLF2* mutation, while *MYD88* and *TP53* mutations are found nearly exclusively in cases without *KLF2* mutation [148].

Splenic Diffuse Red Pulp B-Cell Lymphoma

These neoplasms are low-grade small B-cell neoplasms that diffusely replace the splenic red pulp, unlike SMZLs that preferentially replace splenic white pulp. Based on cytologic and immunophenotypic similarity, the term *splenic marginal zone lymphoma, diffuse variant* also has been used for these tumors. Splenic diffuse red pulp B-cell lymphoma is not well characterized and also resembles cases of so-called hairy cell leukemia variant. The current WHO classification considers splenic diffuse red pulp B-cell lymphoma and hairy cell leukemia variant to be closely related and groups these neoplasms into a provisional entity designated splenic B-cell lymphoma/leukemia, unclassifiable [149]. Recurrent chromosomal alterations frequent in SMZL are infrequent in splenic diffuse red pulp B-cell lymphoma, suggesting that these tumors are a different entity [150]. *NOTCH1*, *TP53*, and *MAP2K1* mutations in splenic diffuse red pulp small B-cell lymphoma are associated with disease progression [150].

Mantle Cell Lymphoma

Mantle cell lymphoma (MCL) is defined as follows: "a B-cell neoplasm generally composed of monomorphic small to medium-sized lymphoid cells with irregular nuclear contours and a *CCND1* translocation" [151]. The two constant components of this definition are B-cell lineage and a *CCND1* translocation, usually t(11;14)(q13;q32), whereas the morphologic appearance can be variable. Thus, the definition of MCL is essentially a molecular definition and this approach has lead to reclassification of a subset of small B-cell lymphomas that morphologically were considered other entities, but are now classified as MCL on the basis of the presence of t(11;14)(q13;q32). MCL represents approximately 6% of all NHLs.

It has been suggested that MCL develops along two pathways [152]. In one pathway, classical MCL is usually composed of *IGHV* unmutated or minimally mutated B cells that usually express the transcription factor SOX11. In the other pathway, MCL develops from *IGHV* mutated SOX11 negative B-cells which leads to leukemic non-nodal MCL, usually involving the peripheral blood, bone marrow, and often spleen [152]. These cases are more frequently clinically indolent. Acquisition of additional molecular/cytogenetic abnormalities, e.g., *TP53* mutation or *MYC* translocation, can lead to more aggressive variants of MCL.

Clinically, patients with MCL are usually elderly, and men are affected more often than women, with a male-to-female ratio of approximately 3 to 1 [153, 154]. B type symptoms occur in 30–40% of patients and 70–80% of patients have stage III or IV disease. Most patients present with generalized lymphadenopathy and bone marrow is commonly involved, approximately 60%. An absolute peripheral blood lymphocytosis of more than 4000/mm [3] is infrequent, but low-level involvement of the blood is common when carefully searched for morphologically or if sensitive molecular techniques are used. Relatively low levels of lymphocytosis probably do not influence survival significantly, but overt leukemia has been associated with a poorer prognosis in some studies [155]. Extranodal disease may occur, usually in association with nodal involvement. Patients may present with numerous polyps involving the gastroesophageal tract below the level of the gastrointestinal junction, also known in the literature as *multiple lymphomatous polyposis* of the intestine. Involvement of Waldeyer ring can occur.

During the CHOP therapy era, patients with MCL had a poor prognosis. R-CHOP generally results in a median PFS of 16–22 months [156, 157]. The importance of high-dose chemotherapy followed by autologous stem cell transplant has been established for patients younger than 65 years old [158]. For elderly patients, maintenance therapy with rituximab and bendamustine is reported to bring better outcome

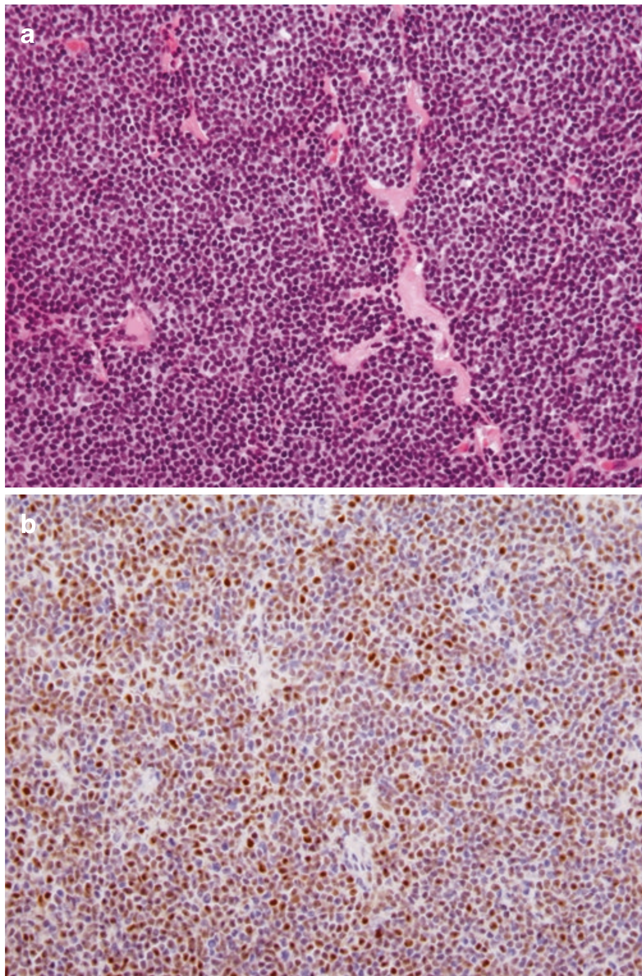

Fig. 40.6 Mantle cell lymphoma involving lymph node. (**a**) The neoplasm is composed of a monotonous population of small cells. Sclerotic blood vessels, common in mantle cell lymphoma, are also shown. (**b**) Cyclin D1 immunostain is positive supporting the diagnosis of mantle cell lymphoma (a. hematoxylin-eosin; b. immunohistochemistry with hematoxylin counterstain, 400×)

[158]. Recently, four agents have been received regulatory approval from FDA for patients with MCL: bortezomib (proteasome inhibitor), lenalidomide (immunomodulator), tensirolimus (mTOR inhibitor), and ibrutinib (Bruton tyrosine kinase inhibitor).

Histologically, MCL replaces the lymph node architecture in a diffuse, nodular, or mantle zone pattern. The mantle zone pattern is rare, occurring as a predominant pattern in less than 5% of cases, and results from the neoplasm selectively involving the follicle mantle zones surrounding germinal centers. Cytologically, typical cases of MCL are composed of a monotonous population of small lymphoid cells that can have slightly irregular or more pronounced irregular nuclear contours (Fig. 40.6) [159]. Large nucleolated lymphoid cells are rare. Other histologic findings common in MCL include numerous eosinophilic epithelioid histiocytes and germinal centers completely surrounded by tumor without a normal lymphoid cuff (so-called naked germinal centers). Plasmacytoid differentiation is rare but can occur in MCL.

Histologically aggressive variants of MCL occur and these can be divided into two types: blastoid and pleomorphic [151]. Blastoid MCLs are characterized by slightly larger lymphoid cells with finely dispersed nuclear chromatin and numerous mitotic features that resemble lymphoblastic lymphoma. Pleomorphic variants of MCL are composed of large cells that resemble, in part, DLBCL. In the blood, there is an aggressive variant of MCL that can resemble B-cell prolymphocytic leukemia. This variant is rare and probably fits within the spectrum of the pleomorphic variant. Histologically aggressive variants of MCL have a more aggressive clinical course, and are independent prognostic factors indicating shorter OS and PFS [160]. In patients who have both typical and pleomorphic variant MCL, either simultaneously of sequentially, clonal identity has been shown in most of the few cases analyzed [161].

Immunophenotypic studies have shown that typical cases of MCL express monotypic Ig light chain (more often Igλ), IgM, IgD, pan-B-cell antigens, Bcl-2, and CD5, and are negative for CD3, CD10, CD21, CD23, CD103, CD200, Irf-4/Mum-1, and Bcl-6 [151]. SOX11 expression has been demonstrated in more than 90% cases of MCL [162]. Using flow cytometry immunophenotyping, unlike cases of CLL/SLL, MCLs typically express brighter surface Ig and CD20, and are usually positive for CD79B and FMC-7. Although CD23 is usually negative in MCL, in 10% of tumors CD23 can be expressed, usually with dim intensity [163]. Cases of MCL can show variant immunophenotypes, but are still recognizable as MCL because they carry t(11;14) and overexpress cyclin D1. Approximately 5% of cases of MCL do not express CD5, or less often can be positive for CD10, Bcl-6, or Irf-4/Mum-1 [151]. In our experience, variant immunophenotypes are more common in histologically aggressive variants of MCL. Ki-67 index is an independent prognostic factor [160].

Conventional cytogenetic analysis of MCL cases has shown that the t(11;14)(q13;q32) involving *CCND1* and *IGH* is present in virtually all cases. This abnormality, however, is present as a sole cytogenetic abnormality in less than 10% of cases [164]. Usually a number of abnormalities are present in MCL and complex karyotypes (≥ 3 abnormalities) are very common. Most of these additional abnormalities include deletions and chromosome copy number changes. Deletions of loci at chromosome 9p21 (*CDK2Na/TPI6*) or 17p (*TP53*) occur relatively often, in up to 20% of cases, and are found mostly in histologically aggressive variants of MCL. Additional balanced chromosomal translocations are uncommon, but t(8;14)(q24;q32)/*MYC-IGH* occurs in a small subset of cases of blastoid variant MCL [165].

MCL has been a subject of intense molecular research in the past decade. A number of high throughput methods have been used to study MCL. These methods have shown that MCL cases have a characteristic, relatively homogeneous gene expression profile. Perhaps this is not unexpected since the definition of MCL is, in large part, based on the t(11;14). These studies also highlighted the importance of proliferation in MCL and how this signature can be used to predict prognosis [166, 167]. Subsequent immunohistochemical studies also have shown that Ki-67 cell counting (proliferating cells) also can be used to predict prognosis in MCL patients treated using a variety of therapeutic regimens [154, 168, 169]. Other methods have shown that there are numerous chromosomal gains and losses, specific deletions, and mutations in cases of MCL. As has been reviewed elsewhere, most of these changes impact the proliferation and cell cycle pathways in the neoplastic cells [170]. Apoptotic mechanisms are also dysregulated in MCL [171].

MCL has mutations affecting many different genes, with *ATM* (40–75%) and *CCND1* (35%) the most frequent [172, 173]. Other mutations are present less frequently, including as *NOTCH1*, *NOTCH2*, *KMT2D*, *WHSC1*, *RB1*, *POT1*, and *SMARCA4*.

Cyclin D1-Negative MCL

In the gene expression profiling study by Rosenwald and colleagues [166], a small subset of lymphomas were identified that morphologically and immunophenotypically resembled MCL, but lacked the t(11;14) as well as cyclin D1 expression. It was suggested these tumors were truly MCL in which aberrant cyclin D2 or cyclin D3 expression was fulfilling the usual role of cyclin D1, hence the designation as cyclin D1-negative MCL. It has become clear that cyclin D1 negative MCL is rare; about 50% of cases carry cyclin D2 translocations detected by FISH or overexpress cyclin D2 shown by RT-PCR [174].

Diffuse Large B-Cell Lymphoma

The diagnosis of DLBCL is based on histologic and immunophenotypic criteria; the tumor is composed of large B-cells with a diffuse growth pattern [175]. DLBCL is the most common type of NHL representing up to 40% of all cases worldwide [175].

In the WHO classification, attempts are made to tease out subgroups of DLBCL on the basis of unique histologic features, distinctive immunophenotypes, or relatively unique clinical presentations (e.g., primary mediastinal large B-cell lymphoma). Cases of DLBCL that cannot be further specified into one of these subgroups are designated as DLBCL not otherwise specified (NOS).

Diffuse Large B-Cell Lymphoma, NOS

DLBCL NOS (henceforth specified as DLBCL) occurs mainly in adults, with a median age in the seventh decade [175–177]. Men are affected slightly more often than women. Although less common in children, DLBCLs represent 15–20% of childhood NHLs. There are differences in immunophenotype and frequency of molecular alterations depending on the geographic region in which DLBCL is being studied. DLBCL can arise *de novo*, or as a transformation event in a patient with low-grade lymphoma, including CLL/SLL, follicular lymphoma, and marginal zone lymphoma.

Patients with DLBCL often have B type symptoms and present with a large, growing mass, often associated with necrosis. Nodal presentation is most common, but extranodal sites are frequently involved, and the disease may be restricted to extranodal sites in up to 40% of patients. A wide variety of extranodal sites can be involved by DLBCL. Approximately half of patients have stage I or II disease. Bone marrow involvement occurs in approximately 20–30% of patients [178]. Two main patterns of bone marrow involvement occur: bone marrow involvement may be concordant in which the cells infiltrating the marrow are large (large cell lymphoma), and this variant is generally associated with poorer OS and PFS [179]. In other cases the lymphoma cells infiltrating the marrow are small and characteristic of low-grade lymphoma (so-called discordant histology). Peripheral blood involvement by large neoplastic cells can be identified in a subset of patients, usually in those patients who have bone marrow disease [175].

If untreated, patients with DLBCL usually die of disease, often within 2 years, however approximately 60% of patients with DLBCL respond well to chemotherapy and can be cured. For years, the mainstay of chemotherapy was the CHOP regimen, but within the past decade rituximab (R) has been added to CHOP and R-CHOP is currently the preferred frontline therapy [180]. Predicting which patients will respond to therapy, however, remains challenging. To address prognostication of DLBCL patients, the International Prognostic Index (IPI) was established in 1993 [181]. The IPI employs five variables, each of which is worth one point: age > 60 years, poor performance status, stage III or IV disease, ≥2 extranodal sites of disease, and above normal serum LDH level. An IPI score of 0–1 indicates good prognosis whereas as an IPI score of 4–5 predicts a poor prognosis and a likely need for more aggressive therapy than the R-CHOP regimen. More recently, improvements to the IPI have seen suggested to further stratify patients. Although very helpful, the components of the IPI are essentially surrogates of DLBCL biology and it seems likely that in-depth knowledge of DLBCL pathogenesis will lead to better

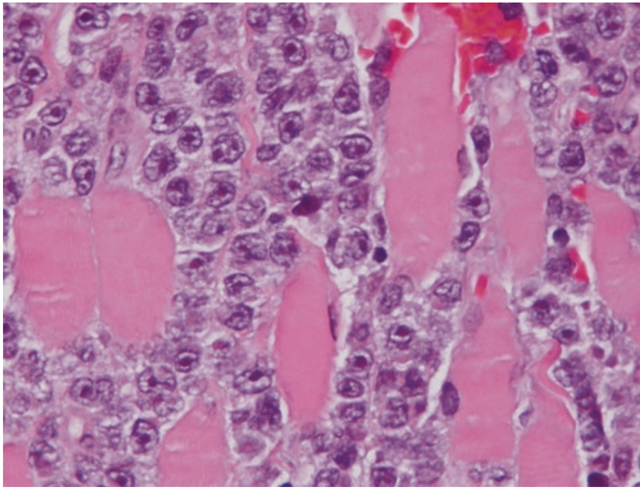

Fig. 40.7 Diffuse large B-cell lymphoma, immunoblastic variant, infiltrating skeletal muscle (hematoxylin-eosin, 1000×)

methods for predicting prognosis as well as identifying specific pathways or molecules for targeted therapy.

Histologically, DLBCLs partially or totally replace normal architecture in a diffuse pattern [175, 176]. Less commonly, DLBCL can partially replace organs, and in lymph nodes, DLBCL rarely can be confined to the interfollicular regions or sinuses. The definition of a large cell is based on nuclear size which needs to be greater than that of a benign histiocyte nucleus; histiocytes are virtually always present admixed within lymphomas. Mitotic figures are usually numerous.

Cytologically, the neoplastic cells in DLBCL can exhibit a spectrum of findings. The most common cytologic variants are centroblastic, immunoblastic (Fig. 40.7), and anaplastic [175]. Centroblasts are 20–30 μm in size and have round or oval vesicular nuclei with two to three nucleoli and more abundant amphophilic cytoplasm. Often one nucleolus is centrally located with one to three additional nucleoli opposed to the nuclear membrane. The term *immunoblast* refers to an activated lymphocyte. Neoplastic immunoblasts resemble transformed lymphocytes; they are larger than centroblasts with a centrally located vesicular round or oval nucleus containing a prominent target-like central nucleolus, and relatively abundant amphophilic cytoplasm. These cells commonly exhibit plasmacytoid differentiation. Anaplastic cells are often very large oval or polygonal cells with pleomorphic nuclei that can resemble, in part, Reed-Sternberg or Hodgkin cells.

Earlier studies of patients with DLBCL showed that patients with the immunoblastic variant had a worse prognosis [182]. However, problems in reproducibly subcategorizing cases of DLBCL lead the WHO classification to group these variants as DLBCL. A recent study has shown that the immunoblastic variant of DLBCL is associated with a poorer prognosis and a high frequency of *MYC* rearrangements, approximately 33% of cases [183, 184].

Approximately two-thirds of cases of DLBCL express monotypic surface Ig light chain, and IgM is the most common Ig heavy chain expressed [175]. Plasmacytoid DLBCLs commonly express monotypic cytoplasmic Ig. Approximately one-third of DLBCLs are Ig negative. DLBCLs express pan-B-cell antigens, 60–70% express Bcl-2, and a subset is positive for CD10, CD23, Bcl-6, or Ifr-4/Mum-1 [175]. DLBCLs are usually negative for T-cell antigens, but approximately 10% of cases can be CD5+. Patients with CD5+ DLBCL are reported to have more aggressive disease [185, 186]. Most cases of DLBCL express activation markers and have a substantial proliferation rate. Major histocompatibility complex (MHC) class I or II antigens can be absent in a subset of DLBCLs; these cases are reported to have a poorer prognosis [175]. CD30 is expressed by a subset of DLBCL and in most cases of the anaplastic variant. Approximately 2% of DLBCL express cyclin D1 [187]. Most cases of cyclin D1+ DLBCL have centroblastic morphology and a post germinal center phenotype positive for Irf-4/Mum-1 [187]. Overexpression of Myc can be detected by IHC in DLBCL cases without a *MYC* translocation [188]. Most studies use a cutoff of 40% or 50% Myc expressing cells to define these cases. In several studies, patients with DLBCL with overexpression of Myc and Bcl-2 protein, so-called double espressor DLBCL, have a poorer prognosis, but not as aggressive as double hit DLBCL with rearrangements of *MYC* and *BCL2* and/or *BCL6* [189].

Gene expression profiling using cDNA microarrays has made its greatest clinical impact in DLBCL. These studies have shown that DLBCL is heterogeneous and can be subdivided into prognostic subgroups [190, 191]. The most popular system divides DLBCL cases into germinal center B-cell like (GCB) and activated B-cell-like (ABC) groups [191]. Patients with GCB tumors have a better prognosis, independent of the IPI, in patients treated with either CHOP or R-CHOP chemotherapy regimens. Virtually all cases of DLBCL with t(14;18)(q32;q21) fall within the GCB group. NF-κB pathway activation has been found to be more prominent in the ABC type [192]. Chronic active B-cell receptor signaling also has been shown in the ABC type [193]. It should be noted, however, that a subset of DLBCLs cannot be classified as either GCB or ABC type, up to 30% in some studies. The GCB and ABC subgroups differ in their chromosomal alterations, activation of signaling pathways, and clinical outcome. Thus, the current WHO classification requires the identification of these two subtypes. As gene expression is not yet readily available for routine diagnosis, a number of groups have suggested various immunohistochemical staining algorithms to predict the GCB versus non-GCB types of DLBCL. One of the most widely accepted is known as the Hans classifier of DLBCL [194]. According to

this scheme cases that are CD10+ in more than 30% of the cells are of GCB type. Cases that are CD10 negative and BCL6+, Irf-4/Mum-1 negative are also in the GCB group. Cases that are Bcl-6-, CD10- or Bcl-6+ and Irf-4/Mum-1+ are in the non-GCB group. There are a number of other competing algorithms for immunohistochemical categorization of DLBCL into GBC or non-GCB types. Two of these systems use FOXP1; FOXP1 and GCET1 by Choi et al. and FOXP1 by Visco et al. [195, 196]. In a multi-center study performed by the Cancer and Leukemia Group B using dose-adjusted EPOCH-rituximab (R) for previously untreated DLBCL, significantly improved time to progression, event-free survival, and overall survival were noted in the GCB group as defined by the Hans method [197]. The efficacy of dose-adjusted EPOCH-R on GCB-DLBCL may relate to its effect on Bcl-6. Although most reports find that immunohistochemical algorithms correlate with prognosis in DLBCL, there are contrary papers arguing that these methods are not reliable surrogates for molecular testing.

Cytogenetics and molecular studies have shown that approximately 50% of DLBCLs carry chromosomal translocations. The most common translocation, in 20–30% of cases, involves chromosome 3q27, the site of the *BCL6* gene [175, 176]. The most common specific translocation is t(3;14)(q27;q32) but there are many others partners of *BCL6* that can be involved in translocations. Another subset of 10–20% of DLBCL cases carries the t(14;18)(q32;q21) involving the *BCL2* and *IGH* genes. The *MYC* oncogene is rearranged in approximately 10% of DLBCLs [175]. These cases are frequently associated with *BCL2*, or to a lesser extent, *BCL6* translocation. These tumors have been referred to as "double hit" or 'triple hit" lymphomas [198–200]. In the revised 2016 WHO classification, these tumors are placed in the new category of high-grade B-cell lymphoma with rearrangements of *MYC* and *BCL2* and/or *BCL6* [17]. In our experience, *MYC* rearrangements are more common in cases with evidence of high proliferation, such as a starry sky pattern or high numbers of Ki-67+ cells. *MYC* rearrangements may involve non-IG or IG partners, most often t(8;14)(q24;q32) involving *IGH*. IG partners convey a poorer prognosis [201]. *MYC* rearrangements are associated with high p53 expression and predict a poorer prognosis even without *BCL2/BCL6* translocation [202].

The IG are rearranged in DLBCL and the IG variable region genes are commonly mutated. *TP53* mutations occur in approximately 15% of DLBCLs and predict prognosis; mutations located specifically in the DNA binding domain of *TP53* are associated with the worst prognosis [203]. *BCL2* is amplified in approximately 20% of cases. Deletions in the *FAS* death domain are reported in approximately 20% of cases, resulting in defects in the extrinsic apoptotic pathway. Other genes that can be mutated in DLBCL with some frequency include *BCL6*, *MYC*, *PAX5*, *PIM1*, and *COK2NA* [204].

Recent molecular studies have identified common somatic mutations in DLBCL but a profile of alterations differentially represented in GCB and ABC subtypes [204]. Somatic mutations common in GCB and ABC DLBCL subtypes are inactivating mutations of *TP53* and genes involved in immunosurveillance (*B2M, CD58*), [204]. GCB-DLBCL carry frequent alterations in chronatin modulating ganes including *CREBBP/EP300, KMT2D/C MEF2* and *EZH2*, *BCL2* translocations, and mutations in the cell motility regulator *GNA13*. ABC-DLBCL have mutations in genes activating the B-cell receptor and Toll-like recepter and NF-κB pathways (*MYD88, CD79A, CARD11, TNFAIP3*) [204]. Mutations in *TP53, FOXO1, MLL3 (KMT2C), CCND3, NFKBIZ,* and *STAT6* have emerged as top candidate genes implicated in therapeutic resistance [205].

T-Cell/Histiocyte-Rich Large B-Cell Lymphoma

T-cell/histiocyte-rich large B-cell lymphoma (TCHRLBCL) is a histologically distinctive type of DLBCL in which the neoplastic large B-cells are few and are associated with numerous reactive T-cells and histiocytes in the biopsy specimen. No cutoff for the number of large B-cells is provided in the WHO classification but <10% is a helpful guideline; B-cells should not be present in aggregates or sheets [206].

Patients with TCHRLBCL tend to be adults with a slight male predominance [207, 208]. Patients often have B-type symptoms and advanced stage disease involving lymph nodes, bone marrow, liver, and spleen. Patients are often refractory to therapy and the disease can resemble DLBCL NOS in relapse biopsy specimens. Some cases of TCHRLBCL may arise from underlying nodular lymphocyte predominant Hodgkin lymphoma (NLPHL).

Histologically, TCHRLBCLs have a diffuse pattern and are composed predominantly of small reactive T-cells and benign non-epithelioid histiocytes [206, 208]. Granulocytes and plasma cells are absent. Usually <5–10% of all cells are singly scattered large, neoplastic B-cells than can appear as centroblasts, immunoblasts, pleomorphic Reed-Sternberg-like cells or lymphocyte predominant (LP)-like large cells. Immunohistochemical studies have shown that the large cells express pan-B-cell antigens, often express Bcl-2 and Bcl-6, and are negative for CD15 and CD138 [206]. The small lymphocytes and histiocytes express T-cell and histiocyte-associated markers, respectively. CD30 can be expressed in a subset of cases.

The large B-cells in TCHRLBCL carry IG gene rearrangements and the IG variable regions genes are commonly mutated. Comparative genomic hybridization has shown

some genetic imbalances, most commonly gains involving chromosome loci Xq, Xp21q11, 4q13q28, and 18q21, and loss of 17p [209]. Gene expression profiling studies have shown that these tumors have a host response signature, which is in keeping with the morphologic and immunophenotypic findings [210].

Differential Diagnosis from NLPHL

In contrast to the indolent clinical behavior of NLPHL, TCHRLBCL is often diagnosed in advanced clinical stages and has a poor prognosis. However, there are variants of NLPHL with histopathologic overlap with TCHRLBCL, especially THRLBCL-like NLPHL, and in small biopsies, it may not be possible to distinguish TCHRLBCL from NLPHL. Gene expression profiling of microdissected tumor cells of NLPHL, TCHRLBL-like NLPHL, and TCHRLBCL have not shown consistent differences in the gene expression profiles of these three entities. Array comparative genomic hybridization of these entities has shown several common and few unique genomic aberrations in NLPHL, TCHRLBL-like NLPHL, and TCHRLBCL, further supporting a close relationship of these entities [9]. Treetipsatit et al. [211] studied 195 cases of NLPHL and TCHRLBCL cases, and found five cases that were difficult to classify according to the current WHO criteria; these cases had a prominent nodular growth pattern with no FDC meshwork. Further refinement of diagnostic criteria is necessary for appropriate classification and clinical management [211].

Lymphomatoid Granulomatosis

Lymphomatoid granulomatosis (LyG), originally described by Liebow and colleagues [212], was originally thought to have features that overlap with malignant lymphoma and Wegener's granulomatosis, leading to its name. LyG is now considered to be neoplastic at onset, is histologically distinctive, is associated with EBV infection, and therefore is included as a type of DLBCL in the WHO classification [213]. LyG is a rare disease.

Patients with LyG are usually middle aged adults (median age 46 years) and there is a male predominance (M:F = 2.2:1) [213]. There appears to be an increased frequency of LyG in immunodeficient patients. Patients present with pulmonary and systemic symptoms and chest radiographs typically show discrete, round masses, most often bilateral [213]. Clinically, all patients have lung involvement (100%), with the next common site being the central nervous system (38%). Other sites that can be involved frequently by LyG include the kidney, liver, and skin. The upper aerodigestive tract is rarely involved [213].

Histologically, LyG is characterized by an angiocentric and angiodestructive infiltrate of many small reactive T-cells with large atypical B-cells, plasma cells, and histiocytes [212, 213]. Granulocytes are rare. Necrosis can be prominent. The large lymphocytes, which represent the malignant cell population, express pan-B-cell antigens and commonly carry Epstein–Barr virus (EBV). The large B-cells can be CD30+, but are always CD15-. In the spectrum of DLBCL, both TCHRLBL and most LyG cases are characterized by the relative rarity of the neoplastic B-cell population with overwhelming background of non-neoplastic surrounding T-cells or histiocytes.

The grading of lymphomatoid granulomatosis relates to the proportion of EBV-positive B-cells relative to the reactive lymphocyte background [213]. It is graded as grade 1 (less than 5 EBV-positive cells/HPF); grade 2 (EBV-positive cells usually 5–20/HPF and occasionally up to 50/HPF); and grade 3 (large aggregates of EBV-positive cells). The natural history of LyG is to accrue large cells and evolve into a process that resembles DLBCL NOS [212].

Polymerase chain reaction studies have shown monoclonal immunoglobulin gene rearrangements and monoclonal EBV episomal DNA in high-grade LyG; demonstration of clonality in grade 1 cases is more inconsistent.

Primary Mediastinal Large B-Cell Lymphoma

The WHO classification defines primary mediastinal large B-cell lymphoma (PMLBL) as DLBCL arising in the mediastinum that is of putative thymic B-cell origin [214]. Applying this definition, however, is not always as easy as it seems clear. A major problem is the difficulty in determining whether a neoplasm arises in the mediastinum and/or the thymus versus mediastinal lymph nodes. Therefore, the category is somewhat "dirty" as it is difficult to exclude all cases of nodal DLBCL that present with a mediastinal mass from the category of PMLBL. The problem is further exacerbated by the increased use of fine needle aspiration and needle core biopsy for diagnosis of mediastinal masses. Although these specimens allow the diagnosis of DLBCL, their small size precludes or makes more difficult the observation of certain features highly suggestive of PMLBL. Despite these limitations, the category of PMLBL has unique clinicopathologic, immunophenotypic, and molecular features [214].

Patients with PMLBL are most often young adults, with onset in the fourth decade [215, 216]. There is a female predominance with a male/female ratio of 1 to 2. Patients typically present with a large, bulky (>10 cm) mass that causes local symptoms such as cough, chest pain, and shortness of breath. Approximately two-thirds of patients present with localized disease and approximately one-third of patients may develop superior vena cava syndrome. Patients typically have high serum LDH levels, but low serum levels of

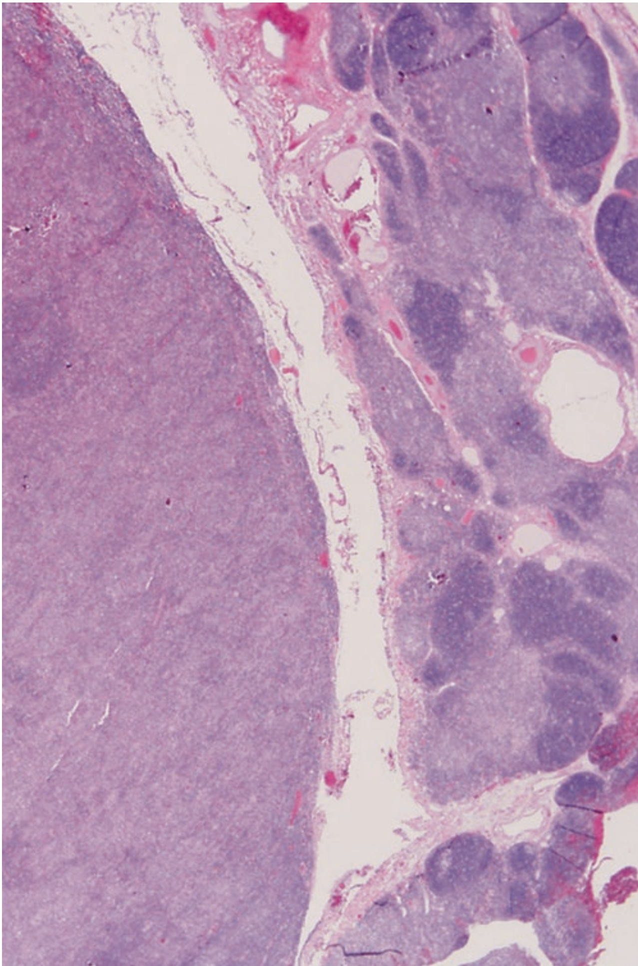

Fig. 40.8 Primary mediastinal (thymic) large B-cell lymphoma. Residual thymus gland is present at the right of the field (hematoxylin-eosin, 20×)

beta-2-microglobulin. Bone marrow involvement or dissemination of disease at the time of diagnosis is absent as, by definition, their presence supports systemic disease [214]. Admittedly, this is a clinical definition that is somewhat arbitrary as patients with PMLBL can theoretically disseminate before first diagnosis of disease. The optimal treatment of PMLBL has not been firmly established. R-CHOP, usually followed by radiotherapy, is the most widely used regimen, but 20–25% of patients still relapse or progress [217]. Better results have been reported with the dose-adjusted EPOCH-R [218]. Relapses commonly occur at extranodal sites, most commonly kidney, liver, adrenal gland, and brain [214].

Histologically, PMLBL diffusely replaces the thymus Fig. 40.8 and invades into contiguous structures including the lungs, pleura, pericardium, and can spread to regional lymph nodes. PMLBL is composed of large lymphoid cells that exhibit a spectrum of cytologic appearances: most commonly resembling centroblasts, and less often resembling immunoblasts, or Reed-Sternberg-like cells [214, 219]. The neoplastic cells often have abundant pale cytoplasm (so-called clear cells). Sclerosis is common and often compartmentalizes the neoplastic cells into groups. Mitotic figures are usually numerous.

Immunophenotypic studies have shown that the neoplastic cells express pan-B-cell antigens and B-cell transcription factors (PAX5, BOB.1, OCT-2, and PU.1) but are commonly negative for surface Ig [220, 221]. About 50% of all PMLBLs lack surface IG [222]. Cyclin E and MAL are commonly overexpressed [221, 223]. Approximately 75% of cases are weakly and/or heterogeneously positive for CD30 and large subsets of tumors are positive for Irf-4/Mum-1, CD23, Bcl-2, or Bcl-6 [214]. T-cell antigens including CD5 are negative as is CD21. PMLBLs show immunophenotypic evidence of NF-κB activation: TRAF1 and nuclear REL. A subset of cases lacks MHC class I or II antigens [214]. MYC does expression by IHC is present in most PMLBLs [224], but not correlate with genetic abnormalities by FISH [224]. CD200 exhibits staining specificity of 93%, similar to that of CD23 (93%) and MAL (97%), and greater than that of TRAF (77%) and REL (83%), suggesting that CD200 is a practical and useful marker for the diagnosis of PMLBLs [225].

PMLBLs carry IG rearrangements and the IG variable region genes are commonly mutated. Balanced chromosomal translocations are uncommon. Comparative genomic hybridization has shown gains at chromosomal loci 2p15, 9q24, Xp11.4–21, and Xq24–26, and losses at 1p13, 4q, 6 of, 7p, and 17p [226]. Recent study with FISH reported that the programmed death ligand (PDL) locus (9p24.1) is frequently and specifically rearranged in PMLBL (~20%) as compared with diffuse large B-cell lymphoma, follicular lymphoma, and Hodgkin lymphoma [227]. Rearrangement was significantly correlated with overexpression of program death ligands 1 and 2 (PDL1, PDL2) [227]. Rearrangements of *CIITA* (MHC class II transactivator) occur in about 25% of cases. A number of genes are amplified in subsets of PMLBL including *JAK2*, *REL*, *BCL11A*, and others. The *BCL6* or *SOCS1* genes are commonly mutated in PMLBL [220, 228]. Both the NF-κB and JAK-STAT pathways are activated in PMLBL, and these tumors also have a host response gene signature.

Gene expression profiling has shown that PMLBL has a signature distinct from GCB or ABC DLBCLs, sharing approximately one-third of its gene expression profile with classical Hodgkin lymphoma [229]. Recently, cases of DLBCL with features of PMLBL by gene expression signature but without detectable mediastinal involvement have been reported [230]. These patients are older, show more equal sex distribution, and show lower frequency of *CIITA* translocations as well as *PDL1/PDL2* abnormalities compared to the PMLBL in mediastinum.

Intravascular Large B-Cell Lymphoma

Intravascular large B-cell lymphoma (IVLBCL), also known as *angiotropic lymphoma* and originally described as malignant angioendotheliomatosis, is a rare variant of DLBCL in which the neoplastic cells grow selectively within blood vessels, especially capillaries [231]. Although the histologic pattern of IVLBCL is quite distinctive, the clinical presentation and immunophenotypes of IVLBCL are strikingly heterogeneous, suggesting that more than one etiology or more than one cell of origin is involved. Rare cases of IVLBCL of T-cell or natural killer cell lineage are also reported, but are not included in this category. The explanation for this pattern of infiltration in IVLBCL is unknown, but the neoplastic cells have been shown to express various chemokines (e.g., CXCR4) or to not express certain types of homing receptors [231, 232]. It has been hypothesized to be secondary to a defect in homing receptors on the neoplastic cells such as the lack of β1 integrin (CD29), and ICAM-1 (CD54) [233].

Patients with IVLBCL are typically middle-aged or elderly, with a slight male predominance, and most patients have B-type symptoms [231, 234]. There are differences in clinical presentation dependent on geographical location. Patients in Western countries tend to present with symptoms attributable to specific organs involved by lymphoma with signs and symptoms related to occlusion of small vessels in various organs, most often the central nervous system and skin. In Asian countries, by contrast, patients often present with widespread disease dissemination, hepatosplenomegaly, and can have cytopenias as a result of hemophagocytic syndrome [231, 234]. Organs commonly involved by IVLBCL include the central nervous system, skin, and kidney but any site may be involved. Standard radiographic studies often falsely do not detect disseminated disease, as no masses are present, but IVLBCL can be detected by FDG-PET scan [235]. Bone marrow involvement, although difficult to appreciate morphologically, is relatively common when the bone marrow is assessed using immunophenotypic or molecular methods [236, 237]. IVLBCL tends to not involve lymph nodes. The prognosis is often poor due to late detection of disease and poor response to chemotherapy [231].

Histologically, IVLBCL is characterized by the presence of large lymphoid cells filling and/or distending vascular lumina [231, 234]. The neoplastic cells are found primarily within capillaries or small blood vessels. Cytologically, the neoplastic cells can resemble centroblasts or immunoblasts. The tumor cells are often associated with tissue hemorrhage or necrosis and fibrin thrombi can be observed. Extravascular involvement can occur in patients with IVLBCL, and although not prominent clinically or radiologically, is not uncommon at autopsy.

With the advent of immunophenotypic studies, IVLBCL was conclusively shown to be a variant of large B-cell lymphoma, most often ABC phenotype. These tumors express pan-B-cell antigens and a subset express monotypic Ig [231, 234]. These tumors commonly express Irf-4/Mum-1, approximately 30–40% of cases are CD5+, and a smaller subset can express CD10. Bcl-6 is usually negative. Proliferation rate, as shown by Ki-67 immunostaining, is usually high.

Molecular studies have shown IG rearrangements in IVLBCL [237]. Rare cases assessed by conventional cytogenetics have shown a complex karyotype.

ALK+ Large B-Cell Lymphoma

This is a pathologically and immunophenotypically distinct type of LBCL in which the neoplastic cells resemble immunoblasts or plasmablasts and express anaplastic lymphoma kinase (ALK) [238]. ALK+ LBCL is a truly rare disease representing <1% of DLBCL cases.

ALK+ LBCL can affect patients of all ages with a male-to-female ratio of 3 to 1 [238, 239]. Lymph nodes are commonly involved and patients tend to present with advanced stage disease, often with involvement of extranodal sites. Histologically, ALK+ LBCL can replace lymph nodes diffusely or exhibit a sinusoidal pattern of involvement or both. The neoplastic cells are large with prominent central nucleoli and abundant cytoplasm resembling either immunoblasts or plasmablasts. The neoplastic cells are usually positive for plasma cell-associated markers (e.g., CD38, CD138), cytoplasmic IgA, and EMA, and can express CD4, CD43 (often focal), CD45 (usually dim if present), CD57, CD79a (often bright), PAX5 (often negative) and Irf-4/Mum-1, and are negative for CD10, CD20, CD30, Bcl-6, T-cell antigens, and EBV. The proliferation rate, shown by Ki-67, is often brisk.

The most characteristic feature of ALK+LBCL is the presence of ALK expression. ALK is expressed as a result of molecular abnormalities involving the *ALK* at chromosome 2p23. The most common abnormality is t(2;17)(p23;q23) involving the *ALK* and *CLTC* genes [238]. Less commonly the t(2;5)(p23;q35) is present in these tumors. The ALK immunostaining pattern correlates with these abnormalities, being cytoplasmic and flocculent in cases with t(2;17) and both nuclear and cytoplasmic in cases with t(2;5). This is attributable to the normal cellular location of the clathrin and nucleophosmin proteins, in cytoplasmic vesicles or the nucleus, respectively [238]. Rare cases are reported with other abnormalities of the *ALK* locus.

Valera and colleagues showed the relevance of the ALK/STAT3 pathway in the pathogenesis of ALK+ large B-cell lymphomas [240]. ALK rearranged receptor binds to ALK-wild type receptor, and triggers STAT3 homodi-

mer formation resulting in phosphorylation of its tyrosine residues. STAT3 dimer can then enter the nucleolus, where it promotes the activation of BLIMP1, a transcription factor that modulates plasma cell differentiation, and *MYC* [240]. BLIMP1, phosphorylated STAT3 and Myc are positive in most cases of ALK+ large B-cell lymphoma [240].

Plasmablastic Lymphoma

Plasmablastic lymphoma (PBL) is defined in the WHO classification as a diffuse, monomorphous proliferation of large neoplastic cells that resemble B-immunoblasts but have an immunophenotype of immature plasma cells [241]. This neoplasm was originally described by Delecluse and colleagues [242] in the oral cavity of patients with HIV infection, but it is now recognized that PBL can occur at virtually any site [241]. Extranodal sites are most often involved. These tumors have been reported in patients with other types of acquired, and congenital immunodeficiency, as well as patients without known immunodeficiency [243, 244]. Many patients have high stage disease and a high IPI score. The prognosis of patients with PBL is poor, despite therapy, with a median survival of approximately 1 year [241, 243].

Histologically, PBL is composed of large neoplastic cells arranged in a diffuse pattern. A starry-sky pattern and a high mitotic rate are common. The neoplastic cells have vesicular nuclei with solitary central nucleoli and abundant cytoplasm with variable plasmacytoid differentiation. Immunophenotypically, the neoplastic cells are positive for CD38, CD138, and Irf-4/Mum-1 and are usually negative for the CD20 and PAX5, although weak expression by a subset of cells can occur in a small subset of cases. The cells of PBL are also commonly positive for cytoplasmic Ig (usually IgG), CD79A, CD30, EMA, and p53, and can express CD10, and CD4. PBLs are usually negative for CD45/LCA, CD3, CD5, and HHV8. CD56 is usually absent and when present should raise the suspicion for plasmablastic myeloma. However in some cases a firm distinction cannot be made between plasmablastic lymphoma or anaplastic plasmacytoma [244]. BLIMP1 and XBP1 have been recently introduced as useful markers for the diagnosis of PBL. The use of a combination of PAX5, CD20, BLIMP1, and XBP1 enables the identification of a plasmablastic immunophenotype highly characteristic of plasmablastic lymphoma cases and associated with an aggressive clinical behavior [245]. The proliferation rate, assessed by Ki-67 expression, is usually high. Myc overexpression was seen in all cases assessed regardless of *MYC* rearrangement status [243]. EBV small encoded RNA (EBER) is detected in 70% of cases. EBV latent membrane protein is negative, consistent with EBV latency pattern 1.

PBL is characterized by acquisition of the transcriptional profile of plasma cells with overexpression of PRDM1/BLIMP1 and XBP1s, in concert with extinction of the B-cell differentiation program with downregulation of B-cell lineage transcription factors such as Pax5 [245]. PBLs carry monoclonal IG rearrangements and the IG variable region genes are often mutated [241]. *MYC* is rearranged in two-thirds of cases [243, 244, 246]. Gene expression analysis identified 645 genes as differentially expressed between PBL and DLBCL, with 257 highly expressed in PBL relative to DLBCL, and 388 lower in PBL [247]. Transcriptional factors *BCL6*, *BCL11A,* and *SPI-B* showed significantly lower mRNA expression in PBLs. A similar trend was observed for CXCR5 and IL4, which have a role in the germinal center (GC) microenvironment. These changes likely reflect post-GC-plasmablastic origin of these tumors. In contrast, mRNA levels of PRMT5, an important transcriptional repressor, were significantly higher in PBLs than in DLBCLs [247]. It seems that the classification of PBL, and its relationship with DLBCL, needs additional study although the clinically aggressive nature of PBL is not in doubt. It is reported that the acquisition of a partial plasmablastic phenotype (BLIMP1 expression) in DLBCL is associated with shorter survival in R-CHOP-treated patients [245].

Primary Effusion Lymphoma

Primary effusion lymphoma (PEL), also known as body cavity-based lymphoma, is a very rare neoplasm of large B-cells that usually involves one of the body cavities: pleural, pericardial, or peritoneal [248]. Usually, only one body cavity is involved. In a subset of patients, PEL also can involve tissue sites as a solid mass and rarely the tumor can initially present as an extracavitary mass. Extracavitary sites are usually extranodal, but we have seen cases of PEL initially diagnosed by lymph node biopsy. Almost all cases of PEL arise in the setting of immunodeficiency, most commonly HIV infection. Rarely, PEL has been reported in patients who have undergone organ transplantation or in elderly patients without obvious evidence of immunodeficiency [248]. HHV8 is universally associated with this lymphoma. In addition, most PEL patients also demonstrate coinfection with EBV, although there is limited expression of EBV-related genes and their role in pathogenesis remains unclear [249]. In 2004, extracavitary or solid variant was proposed for tumors without serous cavities involvement [250]. Classic and extracavitary PEL are similar with regard to morphology, immunophenotype, and molecular characteristics. The prognosis of this lymphoma is poor, with overall

survival commonly less than 1 year despite treatment with high-dose chemotherapy [251].

Morphologically, the neoplastic cells resemble immunoblasts or plasmablasts with prominent nucleoli and abundant basophilic cytoplasm [248, 252]. In some cases anaplastic or Reed-Sternberg-like cells are present. The tumor cells are found within body cavity fluids, but may adhere to and invade body cavity surfaces. In lymph nodes, PEL has a sinusoidal pattern of infiltration. Immunophenotypic studies have shown that PEL cells express CD30, CD38, CD138, EMA, and HLA-DR, and are usually negative for Ig (surface and cytoplasmic), pan-B-cell antigens (e.g., CD19 and CD20) and Bcl-6 [248]. All cases are positive for HHV8 and most cases are positive for EBER and CD45/LCA. A subset of PEL cases can express T-cell associated antigens. Rare cases of "PEL" are reported that lack evidence of HHV8; whether these cases are truly PEL or clinicopathologic mimics is uncertain. The WHO classification suggests that HHV8-negative effusion lymphoma is a distinct entity: lymphoma cells are HHV8 negative and express pan-B-cell antigens, patients are older, and are generally HIV negative, often hepatitis C positive, and often have an underlying medical condition leading to fluid overload [253]. Clinical outcomes and response to therapy in this subset are better than PEL patients. Underlying fluid overload status raises the possibility that these lymphomas are in fact secondary to chronic serosal stimulation [253].

Molecular studies have shown monoclonal IG rearrangements and a subset of cases also has TCR gene rearrangements (lineage infidelity) [248]. *MYC* translocations have been reported rarely. Comparative genomic hybridization has shown frequent gains of chromosome 12p [254]. One gene expression profiling study showed a common gene signature in PEL cases that was distinct from other types of NHL including HIV-associated NHLs. This signature shares features shared with EBV+ transformed lymphoblastoid and plasma cell myeloma cell lines [255].

Burkitt Lymphoma

Clinically, Burkitt lymphoma (BL) cases can be divided into three groups: endemic, sporadic, and immunodeficiency-associated. BL also can present in leukemic phase with preferential involvement of peripheral blood and bone marrow.

Endemic BL was first described by Denis Burkitt in equatorial Africa (Uganda) where it occurs within 15 degrees latitude north or south of the equator [256]. Other endemic areas include Papua New Guinea and Northern Brazil. Evidence of EBV infection is present in over 95% of patients. There are links with malaria and arbovirus infection [257]. The median age of patients with endemic BL is in the range of 4–7 years, with a boy/girl ratio of 2 to 1. The jaw is the most well-known site of disease, involving either the maxilla or mandible in 50–60% of patients, but large abdominal masses involving retroperitoneal structures, the gastrointestinal tract, or the gonads are also commonly present [256, 258].

Sporadic BL occurs in industrialized nations and represents approximately 1% of all NHLs in adults [258]. In children, sporadic BL is the most common type of NHL, up to 40–50% of all cases of NHL. Approximately 20% of patients have evidence of EBV infection. Patients are usually in the second or third decades of life, with a male/female ratio of 2–3 to 1. The jaw is infrequently involved and most patients present with large abdominal masses, frequently involving the ileocecal region of the gastrointestinal tract. Bone marrow and central nervous system involvement are uncommon at the time of presentation, in approximately 10–20% of cases, but are frequent sites of disease later in the clinical course.

Burkitt lymphoma also occurs in the clinical setting of immunodeficiency, most often HIV infection. Immunodeficiency-associated BL is associated with EBV infection in 30–40% of cases, and most often involves extranodal sites [258]. As is the case for all types of BL, EBV within tumor cells is episomal, consistent with latent infection, often present in multiple copies per cell, and monoclonal, indicating that the virus is present prior to neoplastic transformation. However, as is evident by the lower frequency of EBV in sporadic and immunodeficiency BL, EBV alone is not sufficient to cause BL, and neither EBV infection nor *MYC* translocation seems to be sufficient to initiate and maintain neoplastic proliferations in BL. Transforming genes, such as *LMP1*, which are associated with EBV latency, are not expressed in BL cells.

As BL is most often extranodal, and patients commonly present with a high tumor burden and bulky disease, traditional staging systems (e.g., Ann Arbor system) are of less prognostic value than schemes that assess tumor burden and amenability to surgical excision [259]. Patients with BL have a high risk of central nervous system involvement. Waldeyer ring and the mediastinum are involved uncommonly [258]. Since BL is often widely distributed at the time of presentation and has an extremely rapid clinical course, systemic chemotherapy is the treatment of choice. Improvements in diagnosis and combination chemotherapy with CNS prophylaxis have resulted in, more than 90% survival for all-stage children with BL, and for adults 60 years or younger with performance score 0–1 [260, 261]. Patients with immunodeficiency-associated BL also respond well to combination chemotherapy, providing their underlying immunodeficiency syndrome allows adequate therapy to be administered.

Histologically, the endemic, sporadic, and HIV-associated types of BL are very similar and are characterized by two essential features: the neoplastic cells have

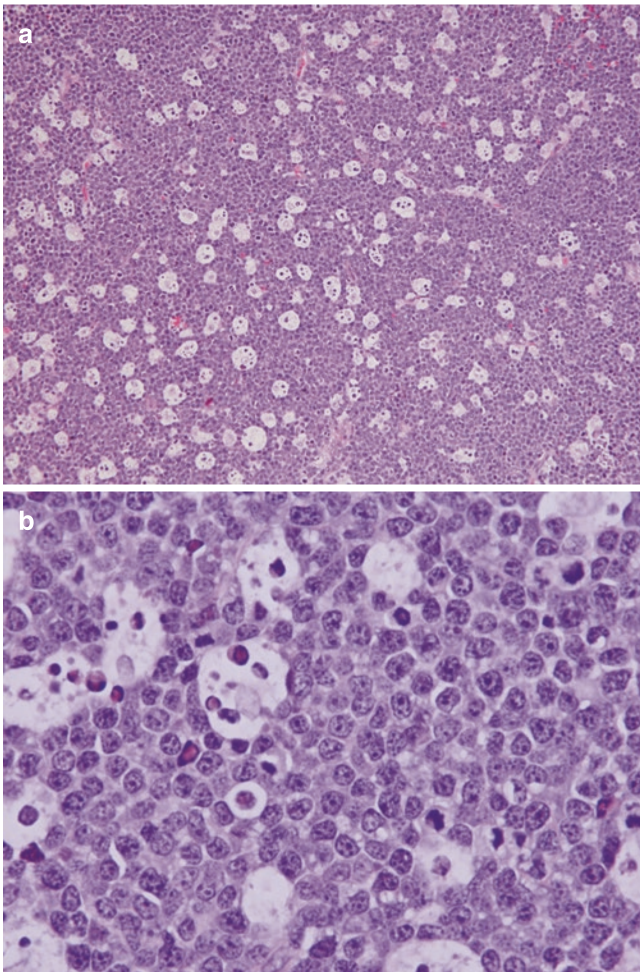

Fig. 40.9 Burkitt lymphoma. (**a**) Low-magnification shows the characteristic starry sky pattern with the histiocytes appearing as clear spaces or "stars" in a background "sky" of darker lymphoma cells. (**b**) The lymphoma cells are of intermediate size with multiple small nucleoli (hematoxylin-eosin, (**a**) 200×; (**b**) 1000×)

nuclei that approximate the size of the nuclei of benign histiocytes and the tumor has extraordinarily high mitotic and proliferation rates [258]. At low power, the neoplasms grow as expansile masses that diffusely infiltrate contiguous tissues. Reactive histiocytes are scattered throughout the tumor. The relatively clear cytoplasm of the histiocytes in a background of blue neoplastic cells imparts a "starry-sky" appearance (Fig. 40.9). This pattern results from rapid cell turnover with individual cell necrosis and scavenging of debris by macrophages. The neoplastic cells are round to ovoid, strikingly monotonous, and uniform in shape. The nuclear membrane is prominent, and the chromatin is coarse, with two to five prominent, basophilic nucleoli. Mitotic figures are numerous. Although the growth pattern of the tumor is usually diffuse, occasionally selective involvement of germinal centers in partially effaced lymph nodes imparts a nodular pattern.

Immunophenotypic studies of endemic, sporadic, and AIDS-associated BLs have shown similar findings. These tumors are of mature B-cell lineage and typically express monotypic Ig light chain, IgM, pan-B-cell antigens, CD10, CD38, Bcl-6, and SOX11. Ki67 proliferating index is 100% in nearly all cases. These tumors are usually negative for IgD, CD21, CD23, CD25, T-cell antigens, TdT and Bcl-2 [258]. Immunophenotypic variants have been reported, however, with a subset of cases Irf-4/Mum-1 and minor subsets negative for CD10, Bcl-6, or Ig, or weakly positive for Bcl-2 [262, 263].

Approximately 80% of cases carry the t(8;14)(q24;q32), with the remaining cases having one of two variant translocations: 15% carry t(2;8)(p11;q24) and 5% carry t(8;22)(q24;q11). Common to each of these translocations is involvement of chromosome region 8q24, the site of the *MYC*. Via these translocations, *MYC* is juxtaposed with the *IGH* on the derivative chromosome 14, or with the *IGK* and *IGL* juxtaposed with *MYC* on the derivative chromosome 8 [264]. *MYC* has a central role in normal cellular proliferation. This juxtaposition of *MYC* and immunoglobulin enhancers results in deregulation of the *MYC* gene, with increased Myc protein driving cell proliferation [265]. *MYC* mutations also occur in BL and may enhance tumorigenicity.

Burkitt lymphomas have IG rearrangements and the IG variable region genes are commonly mutated. Additional cytogenetic abnormalities also occur in BL. In one study, abnormalities of chromosome 13q and 22q were associated with worse prognosis in children, and abnormalities of chromosome 17 correlated with worse prognosis in adults [266]. There was no significant difference in karyotype complexity between children and adults with BL [266]. Frequent copy number abnormalities have also been detected by array-based single nucleotide polymorphism analysis [267]. In general, the karyotypes of BL are simpler than those observed in DLBCL or high-grade B-cell lymphomas (see below) [258].

The breakpoints of the t(8;14) are distinctive in endemic and sporadic BL [264]. In endemic BL, the breakpoint on chromosome 8 occurs far 5′ to the location of *MYC* whereas the breakpoint on chromosome 14 usually occurs within the joining region of *IGH*. By contrast, in sporadic BL, the breakpoint on chromosome 8 occurs within the *MYC* gene, usually in the first exon, or in adjacent flanking sequences. The breakpoint on chromosome 14 may occur within the *IGH* switch regions. These differences suggest endemic and sporadic BL arise at different stages of B-cell differentiation, and may involve mistakes that occur as a result of somatic mutation or *IGH* switching, respectively.

Two major studies of BL using gene expression profiling were published in 2006 [268, 269]. These studies, which focused on sporadic BL, showed that BL has a distinctive

gene expression profile, showing a high level of expression of Myc and germinal center B-cell genes, and a low level of NF-κB. This profile was also identified in a small subset of cases that lacked *MYC* translocations as well as a small subset of cases that resembled DLBCL. In the study by Hummel and colleagues [268], the authors emphasized that there is a spectrum of gene expression that occurs between DLBCL and BL. In both studies, rare lymphomas that carried both *MYC* translocations and t(14;18)(q32;q21)/*IGH-BCL2* translocations were identified that had a gene expression profile of BL and yet did not behave as do most patients with BL [268, 269]. In restrospect, these "double hit" lymphomas should not have been included in the BL category. *MYC* also regulates microRNA expression, including up to 60 microRNAs [265]. Despite differences in epidemiology, endemic, sporadic, and HIV-related BL have been shown to have identical miRNA profiles [270].

Recent molecular studies have improved our understanding of the pathogenesis of these tumors. Mutations in the transcription factor *TCF3* (*E2A*) or its negative regulator *ID3* occur in about 70% of sporadic and immunodeficiency-related BL and 40% of endemic BL [271, 272]. *ID3* mutations are more common than *TCF3* mutations (68% and 11%, respectively), and are also biallelic, suggesting a possible tumor suppressor function [271]. These proteins play an important role in germinal center B-cell differentiation, thus dysregulation of this signaling pathway seems important in Burkitt lymphomagenesis [273]. The interplay between EBV infection and Burkitt lymphomagenesis still remains unknown.

Burkitt-Like Lymphoma with 11q Aberration

Some recent studies have identified a subset of lymphomas that resemble BL morphologically, immunophenotypically, and by GEP, but which lack *MYC* rearrangements [274, 275]. Instead they have a chromosome 11q alteration characterized by proximal gains and telomeric losses. Further studies have identified candidate genes potentially affected by these imbalances: *PAFAH1B2*/11q23.3 (overexpressed), *FLI1* (downregulated), and *ETS1* (recurrently mutated) [274, 275]. Compared with classic BL, these lymphomas have more complex karyotypes, lower levels of Myc expression, occasionally a follicular pattern, and frequently present with nodal disease [274, 275]. The clinical course seems to be similar to BL, but the number of cases reported is still limited. These cases are considered as a provisional entity in the revised 2016 WHO classification [17].

High-Grade B-Cell Lymphomas

The terminology "B-cell lymphoma, unclassifiable, with features intermediate between diffuse large B-cell lymphoma and Burkitt lymphoma (BCLU)" was adopted by the 2008 WHO classification for aggressive B-cell lymphomas with morphologic, phenotypic, and genetic features which overlap between Burkitt Lymphoma and DLBCL [276]. The tumors in this category had great overlap with lymphomas designated previously in the International Working Formulation as small noncleaved cell lymphoma, non-Burkitt type, or in the REAL classification as high-grade B-cell lymphoma, Burkitt-like. Later, additional studies demonstrated that B-cell lymphomas with rearrangements of *MYC* and *BCL2* and/or *BCL6* had features intermediate between DLBCL and BL. *MYC* translocations were identified in 35–50% of cases in the entity of B-cell lymphoma, unclassifiable, with features intermediate between diffuse large B-cell lymphoma and Burkitt lymphoma. In WHO 2016 revision, BCLU is re-named and separated in two categories: high-grade B-cell lymphoma with *MYC* and *BCL2* and/or *BCL6* translocations, and high-grade B-cell lymphoma, NOS [17].

High-Grade B-Cell Lymphoma with MYC and BCL2 and/or BCL6 Translocations

This group includes so-called "double hit" or "triple hit" lymphomas, with simultaneous presence of *MYC* and *BCL2* translocations, *MYC* and *BCL6* translocations, or *MYC*, *BCL2*, and *BCL6* translocations. These lymphomas are typically composed of a diffuse proliferation of medium to large sized cells, with irregular nuclear contours, and relatively large nucleoli. Many mitotic figures, prominent apoptosis, and a starry-sky pattern are often present. All large B-cell lymphomas with *MYC* and *BCL2* and/or *BCL6* rearrangements are included in this category [17]. In about 60% of cases the *MYC* translocations involve an IG gene partner, and in 40% of the cases they involve non-IG partners. Immunoglobulin gene partners are associated with a poorer prognosis [201]. This lymphoma occurs in adults (median age 55) and are of GCB phenotype [277, 278]. Double-hit lymphomas with *BCL6* and *MYC* translocations are rare aggressive B-cell lymphomas, tend to occur in old women, are frequently extranodal in presentation, and have a poor prognosis. They can be either GCB or ABC phenotype [279]. Triple-hit lymphomas are rare and aggressive lymphoma. In one study of 11 cases of triple-hit lymphomas, all patients were men, with a median age of 64 years old [200]. All cases were positive for CD10, Bcl-2, and FOXP1, Bcl-6 and Irf-4/Mum-1 were positive in 73% and 55%, respectively. All cases belonged to GCB immunophenotype [200].

High-Grade B-Cell Lymphoma, NOS

Cases that have intermediate features between DLBCL and BL but which lack a *MYC* and *BCL2* and/or *BCL6* rearrangement

are placed in the category [17]. Histologically, these neoplasms are diffuse, often have a starry-sky pattern, and have a high proliferation (Ki-67) rate (>99%) with numerous mitotic figures. The neoplastic cells, however, are of intermediate size similar to BL cells, but with greater nuclear pleomorphism, or are larger with more vesicular nuclear chromatin somewhat similar to DLBCL cells, but not as large. Cases of this category can histologically resemble BL but have atypical immunophenotypic features [17]. For example, these tumors express pan-B-cell antigens, CD10, and Bcl-6 which have a high proliferation (Ki-67) rate, and yet also strongly positive for Bcl-2. Blastoid-appearing large B-cell lymphomas which lack *MYC* rearrangement are also included in this category [17].

B-Cell Lymphoma, Unclassifiable, with Features Intermediate Between Diffuse Large B-Cell Lymphoma and Classical Hodgkin Lymphoma

These neoplasms are defined as having "overlapping clinical, morphological, and/or immunophenotypic features between classical Hodgkin lymphoma and DLBCL" [280].

Most cases designated as B-cell lymphoma, unclassifiable, with features intermediate between diffuse large B-cell lymphoma and classical Hodgkin lymphoma CHL are tumors at the interface between primary mediastinal large B-cell lymphoma (PMLBL) and classical Hodgkin lymphoma (CHL). The median age of patients is 33 years, and about 50% of patients have a mediastinal masses greater than 10 cm [281]. The clinical course of affected patients is thought to be more aggressive than that of patients with either PMLBL or CHL, although recent study suggests that dose-adjusted EPOCH-R produces durable remissions in most patients [281].

Using histologic and immunophenotypic findings, there are essentially two types of B-cell lymphoma, unclassifiable, with features intermediate between DLBCL and CHL. In one type, the neoplasm is composed of sheets of large neoplastic cells resembling PMLBL, but the immunophenotype has features more in keeping with CHL (e.g., CD15+ or EBV+). In the other type, the neoplasm resembles CHL, usually nodular sclerosis type, but the neoplastic cells have an immunophenotype more like DLBCL (e.g., CD45/LCA+, strong expression of B-cell antigens). A subset of these tumors expresses MAL, as is seen in PMLBL. Strong CD20 expression is acceptable in cases diagnosed as CHL, and by itself, is not a reason to classify the neoplasm as a gray zone tumor.

Gene expression profiling of cases of PMLBL and CHL have shown that these neoplasms share aspects of their gene expression profiles [229]. Approximately one-third of the genes expressed in PMLBL and CHL are shared. These data provide, in part, a rationale for the WHO proposal of this category of disease.

T-Cell Non-Hodgkin Lymphomas

The classification of T-cell lymphomas and leukemias is challenging because, unlike B-cell NHLs, T-cell NHLs do not seem to correspond to normal stages of differentiation nor are most tumors associated with chromosomal translocations. As a result, the classification of some types of T-cell lymphoma, particularly extranodal forms, currently incorporates clinical findings into the classification system [17]. Knowledge of T-cell function, likely to emerge over time, may be helpful to more specifically classify T-cell lymphomas and leukemias. In the following sections, the focus is placed on T-cell neoplasms that usually or commonly present as lymphoma.

T Lymphoblastic Leukemia/Lymphoma

T lymphoblastic leukemia/lymphoma can present in two fashions: (1) as acute lymphoblastic leukemia (T-ALL) with extensive involvement of bone marrow and blood; and (2) as lymphoma (T-LBL), most often as an anterior mediastinal mass or lymphadenopathy. Patients who present as T-LBL often convert to a T-ALL clinical picture over time, and many patients who present as T-ALL have an anterior mediastinal mass. This clinical overlap, as well as overlap in immunophenotypic and molecular features, led the authors of the WHO classification to combine T-ALL and T-LBL cases into one category [282]. There are some immunophenotypic and molecular differences between T-ALL and T-LBL; however, that may explain their different clinical presentations [283]. Here we focus on patients presenting as T-LBL.

Patients with T-LBL are most often adolescents and young adults with a male-to-female ratio of 2 to 1 [282, 283]. Patients commonly present with widespread, supradiaphragmatic lymphadenopathy and/or an anterior mediastinal mass. Patients with a mediastinal mass may have dyspnea, cough, stridor or superior vena cava syndrome. Pleural and pericardial effusions are common. Peripheral blood involvement is seen in approximately one-third of patients at the time of presentation. Compared with patients with T-ALL, patients with T-LBL have lower serum LDH levels, a lower frequency of hepatosplenomegaly, and a lesser risk of central nervous system involvement [283]. T-LBL grows rapidly and, unless effectively treated, patients have a rapidly progressive down-

hill course with widespread dissemination of disease. Patients with T-LBL respond favorably to therapeutic regimens designed after those used for ALL.

Histologically, T-LBL replaces the lymph node in a diffuse pattern and often spills through the capsule into perinodal tissues [282, 284]. In cases with partial involvement, lymphoid follicles may be spared. A starry-sky pattern is present in a subset of cases. Rarely the neoplasm is compartmentalized by fibrous tissue imparting a nodular low power pattern. Cytologically, T-LBL is composed of lymphoblasts that have “dusty” or granular chromatin and either round or convoluted nuclear contours. Mitoses are numerous.

Immunophenotypic studies have shown that T-LBLs appear to illustrate the concept that these neoplasms represent clonal expansions of cells “frozen” in a state of differentiation [282]. This phenomenon allows one to deduce that a variety of antigens are acquired in sequence, most likely reflecting the sequence of expression by non-neoplastic lymphoid cells during development. These stages of T-cell differentiation can be divided into early T-precursor, pro-T, pre-T, cortical T, and medullary T [282, 285, 286]. CD7 and cytoplasmic CD3 expression occur very early in this sequence and are expressed in the pro-T stage. The early T-precursor, pro-T, and pre-T stages are CD4-, CD8- and the cortical T stage is CD4+, CD8+ and CD1a+. T-LBLs also commonly express CD34, CD99, and Bcl-2. Myeloid-associated antigens are expressed in up to one-third of cases [282]. T-LBL are negative for Ig and pan-B-cell antigens, with the exception of CD79A which can be positive in a subset of T-LBLs, approximately 50% in one study [287].

Most cases of T-ALL/LBL show TCR gene rearrangement, and there is a hierarchy: *TRD* followed by *TRF*, *TRB* and *TRA*. These cases also exhibit lineage infidelity manifested by the presence of *IGH* gene rearrangements in approximately 20% of cases [282, 288].

Most cytogenetic and molecular studies performed on T-cell lymphoblastic neoplasms have been performed on cases of T-ALL, with relatively few cases of T-LBL assessed. Based on the available data, there is overlap between T-LBL and T-ALL, but it must be noted that the cytogenetic and molecular profile for T-LBL presented here is based primarily on T-ALL data. Conventional cytogenetic studies of T-ALL/LBL have shown abnormalities in approximately 50% of cases [283, 289, 290]. The most common translocations involve the TCR genes at chromosomal loci 14q11 (*TRA* and *TRD*), 7q35 (*TRB*), and 7p14-15 (*TRG*). The most frequent gene partners are *HOX11/TLX1* at 10q24 and *HOX11L2/TLX3* at 5q35, with many other partners including *TAL1* (1p32), *RBTN1/LMO1* (11p15), *RBTN2/LMO2* (11p13), *LYL1* (19p13), and *MYC* (8q24). These translocations are of two types: (1) intact oncogenes that are juxtaposed with TCR gene loci; (2) dysregulated genes that are fused, creating a novel, chimeric gene. Many additional cytogenetic or molecular alterations have been shown in T-ALL, most of which affect the cell cycle. *NOTCH1* mutations are common in T-ALL, in 40–50% of cases, and additional abnormalities in other genes in the *NOTCH1* pathway have been reported, such as loss of *PTEN* and *AKT* activation [291]. Additional somewhat common alterations in T-ALL include: deletions of chromosome 9p21 (30–40%), del6q (in 15–20%), *WT1* mutations (~10%), duplications/gains of chromosome 6q23/*MYB* (5–10%), rearrangements/amplification of *ABL1* (5%), protein tyrosine phosphatase 2 (*PTPN2*) mutations, and deletions of chromosome 13q14/*RB1* [292–295].

Gene expression profiling of T-ALL cases has shown distinct gene signatures that correlate with the known cytogenetic abnormalities [296]. These studies also have shown that T-ALL without identified cytogenetic abnormalities can be characterized by similar gene signatures suggesting the presence of cytogenetically undetectable abnormalities in these pathways. There are at least five gene signature groups that correlate, in part, with immunophenotype: HOX11/TLX1 (early cortical T), HOX11L2/TLX3 (early cortical T), TAL1 plus either LMO1 or LMO2 (late cortical T), LYL1 plus LMO2, and MLL-MLLT1 (pro-T). Comparisons of T-ALL and T-LBL have been few to date: T-LBL cases preferentially express genes encoding cell adhesion and extracellular matrix proteins [296].

Early T-Precursor Acute Lymphoblastic Leukemia (ETP-ALL)

Early T-precursor acute lymphoblastic leukemia (ETP-ALL) is a recently described subgroup of T-ALL with a poor prognosis, originating from a subset of thymocytes that retain stem-cell-like features [286, 297]. This leukemia is defined immunophenotypically by the absence of CD1a, CD4, and CD8 expression, low CD5 (<75% positive cells), and expression (>25% positive cells) of at least one or more of the following myeloid or stem cell markers: CD117, CD34, HLA-DR, CD13, CD33, CD11b, or CD65 [286]. ETP-ALL show unique genetic and transcriptional features that overlap both with T-ALL and AML, and show lower frequencies of prototypical T-ALL lesions such as activating mutations in *NOTCH1* [297]. Cases of ETP-ALL also have a high prevalence of mutations typically associated with the pathogenesis of acute myeloid leukemias including *IDH1, IDH2, DNMT3A, FLT3,* and *NRAS* [297, 298].

Adult T-Cell Lymphoma/Leukemia

Adult T-cell lymphoma/leukemia (ATLL) is a type of mature T-cell lymphoma that is caused by infection by the human

T-cell leukemia/lymphoma retrovirus (HTLV-1) [299]. ATLL accounts for the high incidence of T-cell lymphoma in Japan, particularly on the islands of Kyushu and Okinawa where up to 5–6% of the population are seropositive for HTLV-1. However, the prevalence of seropositivity has decreased from up to 30% of individuals being seropositive for HTLV-1 in the 1970s [300]. Other endemic regions for HLTV-1 infection include Brazil and Caribbean countries and cases are also reported, albeit infrequently, in Europe, and the United States.

HTLV-1 is a single-stranded RNA retrovirus that is lymphotropic for T-lymphocytes [301]. The virus is transmitted by sexual intercourse, breast milk, shared needles among intravenous drug users, and transfusion of blood products. Rare cases of vertical transmission without breastfeeding are also reported [302]. The virus, however, is insufficient to cause lymphoma by itself and therefore other abnormalities must occur. This sequence of events most likely takes years as the incubation period for development of ATLL in HTLV-1 individuals ranges from 20 to 40 years [301].

Patients with ATLL are usually adults, with a median age between 58 and 62 years and a male-to-female ratio ranging from 1.2–1.5 to 1 [299, 303]. Affected patients may present with one of four variants: acute, lymphomatous, chronic, and smoldering [304]. Patients with acute ATLL, the most common form of the disease, have generalized lymphadenopathy, hepatosplenomegaly, skin lesions, peripheral blood involvement with high leukocyte counts, lytic bone lesions, and hypercalcemia. Hypercalcemia also may develop in the absence of bone lesions. The lungs and central nervous system are commonly involved. Patients with the lymphomatous form of ATLL present with prominent lymphadenopathy and tumors in other organs without hepatosplenomegaly or hypercalcemia. Skin lesions are common. Many patients with the acute form of ATLL, and a lesser number with lymphomatous ATLL, have evidence of immunodeficiency and opportunistic infections. These patients respond to chemotherapy, but remissions are short and overall survival is usually poor [305].

Patients who present with chronic variant ATLL have an absolute lymphocytosis and cytologically abnormal cells in the blood. Skin lesions, lymphadenopathy, and involvement of other viscera may occur and survival is usually greater than 2 years [299, 304]. Patients who have the smoldering form of ATLL have chronic disease for years, usually skin lesions with minimal peripheral blood involvement; the viscera are usually spared. Patients with the chronic and smoldering variants of ATLL do not usually require therapy but should be carefully followed as approximately 25% of patients transform to acute or lymphomatous variant ATLL [299, 305].

The neoplastic cells in the peripheral blood smear in patients with acute or lymphomatous ATLL are characteristic [299]. The circulating neoplastic cells are medium-sized with basophilic cytoplasm and markedly irregular, multilobulated nuclei, including cloverleaf shapes, also known as *flower cells*. In lymph nodes and viscera, ATLL diffusely replaces lymph nodes or extranodal sites. Cytologically, ATLL exhibits a spectrum of cell sizes, including small, medium-sized, large, anaplastic, and Reed-Sternberg-like cells [299, 306]. Histologically, lymph nodes may exhibit classical Hodgkin lymphoma or angioimmunoblastic T-cell lymphoma like morphology [299]. Skin lesions are seen in more than 50% of patients with ATLL. Epidermal infiltration with Pautrier-like microabscess is common, which can mimic mycosis fungoides. Many cases are indistinguishable from other T-cell lymphoma subtypes without knowledge of HTLV-I infection.

Immunophenotypic studies have shown that ATLLs have a mature T-cell immunophenotype [299]. The neoplastic cells express the pan-T-cell antigens CD2, CD3, CD5, and the TCR αβ receptor, but are usually negative for CD7. Most cases of ATLL are CD4+ CD8-. The lymphoma cells intensely express the CD25, and approximately 40% of ATLLs are positive for CCR4 and FOXP3; markers of regulatory T-cells [307]. ATLL cells variably express CD30 in a subset of cases, and are negative for Ig and B-cell antigens.

Conventional cytogenetic studies have shown a wide range of abnormalities in cases of ATLL. Usually multiple abnormalities are identified per individual neoplasm, at least six in one study [308]. These results suggest a multistep pathogenesis for ATLL, and are consistent with the known long incubation period from the time of infection to onset of tumor. Several studies with array based comparative genomic hybridization show frequent losses at chromosomes 6q and 13q, frequent gains at chromosomes 14q, 7q, and 3p, as well as aneuploidy (+3, +7, +21, −X, −Y), and translocations involving 14q11 and 14q32 (*TRA* and *TRD*, respectively). Increased number of chromosomal imbalances are associated with a significantly shorter survival [309].

The hallmark for the diagnosis of ATLL is the demonstration of HTLV-1 infection. Molecular identification of HTLV-1 is ideal for this purpose as the virus clonally integrates into the host cell genome in a random fashion [301]. The site of HTLV-1 integration into the genome appears to be similar in most cases; unusual sites of viral integration may correlate with a poorer prognosis [310]. The mechanisms to explain how HTLV-1 is involved in lymphomagenesis are not well worked out. The TAX viral protein and HTLV-1 basic leucine zipper factor (HBZ) are thought to be involved. Another point worth mentioning is that use of serum testing for HTLV-1 infection as a surrogate for molecular evidence of HTLV-1 infection is imperfect. In

countries with a low prevalence of HLTV-1 infection, the presence of HTLV-1 antibody highly correlates with ATLL in a patient presenting with evidence of lymphoma. However, in Japan and other areas with a high prevalence of HTLV-1 infection, antibody testing yields a high false positive rate [311].

Acute and chronic variants of ATLL, differ in their gene expression profile and in chromosome copy number. Expression of MET is high in acute variant ATLL suggesting a potential therapeutic target [312]. Recent study of an integrated molecular analysis of a total of 426 ATLL cases identified alterations of NF-κB signaling [313]. Other notable features include a predominance of activating mutations (*PLCG1, PRKCB, CARD11, VAV1, IRF4, FYN, CCR4, CCR7*) and gene fusions (*CTLA4-CD28* and *ICOS-CD28*) [313]. Frequent intragenic deletions involving *IKZF2*, *CARD11*, and *TP73* and mutations in *GATA3*, *HNRNPA2B1*, *GPR183*, *CSNK2A1*, *CSNK2B*, and *CSNK1A1* were also observed [313].

Extranodal NK/T-Cell Lymphoma, Nasal-Type

Extranodal NK/T-cell lymphoma, nasal type (NK/T-cell lymphoma) was designated previously as lethal midline granuloma, polymorphic reticulosis, midline malignant reticulosis, and angiocentric lymphoma [314]. This neoplasm exhibits marked geographic variation, with a high frequency in Asia and native Americans in Central and South America.

Clinically, NK/T-cell lymphomas have a marked propensity for involving extranodal sites, most commonly the nasal cavity, nasopharynx, and palate [314, 315]. In a subset of patients, the disease involves non-aerodigestive tract sites (so-called non-nasal type) [315]. Extranodal NK/T-cell lymphomas involving the upper aerodigestive tract are predominantly localized processes at onset and may be present for years prior to diagnosis, but widespread dissemination can occur including lymph nodes and bone marrow [314].

Extranodal NK/T-cell lymphomas are commonly associated with a hemophagocytic syndrome, clinically characterized by fever, hepatosplenomegaly, pancytopenia, and laboratory evidence of hemolysis. Based on their extranodal distribution, traditional staging systems are less useful and a system based on the presence of local invasion and stage (localized versus disseminated) has been proposed [316]. The prognosis of patients with NK/T-cell lymphomas involving the aerodigestive tract has improved with chemotherapy and radiotherapy [317, 318]. The prognosis of patients with non-nasal NK/T-cell lymphomas is much poorer [315].

Histologically, NK/T-cell lymphomas are diffuse, destructive tumors that almost invariably are associated with necrosis and commonly exhibit angioinvasion and angiodestruction [314, 319]. The cytologic spectrum of NK/T-cell lymphomas is very wide and neoplasms can be composed of small or very large cells. A prominent inflammatory cell background is also usually present, but eosinophils and neutrophils are rare or absent. In the early stages of disease, relatively few neoplastic cells associated with inflammatory cells can be observed, particularly in a small biopsy specimen, leading to misinterpretation as a benign process [314, 319].

Immunophenotypic studies of NK/T-cell lymphomas have shown that most cases are of NK-cell origin, although a subset of cases are of T-cell lineage [314, 319]. Virtually all cases of NK/T-cell lymphoma are positive for EBER [314]. Most cases also express the NK-cell-associated antigen CD56, and NK/T-cell-associated antigens such as CD2, and CD7. All cases of NK/T-cell lymphomas express cytoplasmic CD3e; NK-cell tumors are negative for T-cell-specific antigens such as surface CD3, CD5, and TCRs, whereas T-cell tumors express these T-cell markers. Most NK/T-cell lymphomas express cytotoxic markers (e.g., perforin, TIA-1, and granzyme B) [314, 320]. The neoplastic cells, when of large size, can express p53, FAS, and FAS ligand [320]. Ki-67 rate correlates, in part, with cell size and prognosis in localized neoplasms [321]. CD16 and CD57 are usually negative, and Ig and B-cell antigens are negative.

Molecular studies of NK/T-cell lymphomas have shown an absence of TCR and IG rearrangements in NK cases, and TCR gene rearrangements in T-cell cases [314]. EBV genomes have been identified in the neoplastic cells using Southern blot, PCR, and in situ hybridization techniques. The virus also has been shown to be present in monoclonal form indicating that EBV is present prior to clonal expansion. Comparative genomic hybridization studies of these neoplasms have shown gains of chromosome 2q and many losses involving chromosomal 1p, 4q, 5q, 6q, 7q, 12q, and 15q [322]. Deletion of chromosome 6q (6q21-6q25) is found in 40–50% of NK/T-cell lymphoma cases. Several putative tumor suppressor genes (*HACE1*, *PRDM1*, *ATG5*, *AIM1*, *FOXO3*) were identified in the 6q21–q25 region, and their roles as a potential tumor suppressors were suggested [323–325]. Many other gene alterations are reported, involving angiogenic genes, platelet-derived growth factor alpha, tumor suppressor genes including *TP53*, and deregulation of the AKT, JAK-STAT, and NF-κB pathways. Among these, *TP53* mutations appear to correlate with large cell morphology and more advanced-stage disease [326].

Enteropathy-Associated T-Cell Lymphoma

Enteropathy-associated T-cell lymphoma (EATL) is a rare and clinically distinctive type of T-cell lymphoma that represents <1% of all NHLs. These tumors are thought to arise from intraepithelial T-cells normally present in the gastrointestinal tract, particularly the jejunum and ileum [327]. This lymphoma

is closely linked to celiac disease. As a result, EATL is more common in Northern Europe, where celiac disease is more common. Patients with EATL present with abdominal pain, weight loss, or complications of intestinal perforation [327, 328]. Most, but not all patients with EATL have a history of celiac disease, usually with onset in adulthood. Despite combination chemotherapy the survival of individuals with EATL is poor [327, 328]. Approximately half of patients initially respond to chemotherapy, but almost all relapse and death results from either disease progression or complications of therapy.

It also should be noted that patients with celiac disease have an increased risk of B-cell NHLs, both intestinal and non-intestinal, and B-cell NHLs are more common than EATL [329]. It seems inconsistent that EATL is recognized by the WHO classification whereas B-cell NHLs in patients with celiac disease are not specifically recognized in a similar manner.

Grossly, the involved intestine demonstrates an ulcerating mass than can be flat or exophytic. The ulcer(s) often extend deeply into the bowel wall resulting in perforation. The jejunum and ileum are the most common sites of involvement, but other sites in the gastrointestinal tract have been reported rarely [327]. Histologically, the contiguous, uninvolved intestine often exhibits blunting of villi, increased plasma cells and lymphocytes in the lamina propria, and crypt hyperplasia: evidence of celiac disease. Cases of EATL diffusely replace the intestinal wall and necrosis is common [327, 330]. The neoplastic cells show a wide cytologic spectrum: small, intermediate-sized, large, anaplastic, or Reed-Sternberg-like. Numerous histiocytes and eosinophils are present in the background.

Immunophenotypic studies have shown that EATL tends to be CD3+, CD5-, CD7+, CD56-, CD103+, TCR αβ, and CD4-. CD8 is expressed in 20% of cases [327]. These tumors often express CD30 variably and usually express cytotoxic molecules. Fifty to sixty percent of EATL are positive for EBV.

Cases of EATL show TCR rearrangements and the IG are in the germline configuration [327]. Molecular analysis of biopsy specimens from patients with refractory celiac disease or ulcerative jejunitis, without morphologic evidence of lymphoma, also has shown TCR gene rearrangements suggesting that these diseases are preneoplastic conditions [331]. Comparative genomic hybridization has shown gains of chromosome loci 1q32, 5q34-q35.2, 9q31.3, and 16q12.1 [327, 332].

Monomorphic Epitheliotropic Intestinal T-Cell Lymphoma (MEITL)

This category is newly recognized in the current WHO classification and includes lymphomas that were previously designated as type II EATL [17, 327]. These tumors show no association with celiac disease, and show geographic distribution with increased incidence in Asians and Hispanic populations [17, 333]. MEITL is composed of a monomorphous intermediately sized population of cells. Necrosis is uncommon and there are fewer admixed inflammatory cells in the background than EATL [17]. MEITL has a T-cell immunophenotype similar to EATL, but usually positive for CD8 and CD56 [17]. Gains in chromosome 8q24 which involves *MYC* are seen in high proportion of cases [333, 334]. Up to 80% of cases express TCR γδ, small subset expresses TCR αβ, and coexpression of TCR αβ and TCR γδ as well as TCR silent cases are also reported [335]. Recent study demonstrated *STAT5B* mutations in 36% of MEITL with TCR γδ expression. *STAT3* mutations which are also observed in other T-cell lymphomas with TCR γδ expression were not observed in MEITL [336].

Hepatosplenic T-Cell Lymphoma

Hepatosplenic T-cell lymphoma (HSTCL) is a rare tumor that represents <1% of all NHLs. These neoplasms most commonly affect young adult men, with a median age of up to 35 years, and a subset of cases, approximately 20%, arise in patients with a history of immunosuppression [337, 338]. Patients with HSTCL commonly present with systemic, B-type symptoms and the complete blood count commonly shows thrombocytopenia, often with anemia and leukopenia [337–339]. Leukemic involvement is uncommon at the time of initial diagnosis. Patients also have marked hepatosplenomegaly and minimal or absent lymphadenopathy. Affected patients have an aggressive clinical course with a high rate of relapse and median overall survival of 28 months. An elevated serum bilirubin level, αβ TCR expression, and trisomy 8 are reported to correlate with poorer outcome [337, 338]. Recently, stem cell transplant has been reported to result in long-term survival for a substantial proportion of patients with HSTCL [338, 340].

Histologically, HSTCL diffusely replaces the red pulp of the spleen and involves sinusoids in the liver and bone marrow [337, 341]. Neoplastic cells of HSTCL are predominantly intermediate in size, with pale cytoplasm and round nuclei with condensed chromatin and inconspicuous nucleoli; however, they also show some cytological spectrum ranging from small-sized cells with high N/C ratio and hyperchromatic nuclei to large cells with irregular nuclear contours [338, 341]. Bone marrow is involved in virtually all cases at the time of diagnosis, and mild dyspoietic changes in the background trilineage hematopoietic cells are often observed [342].

Immunophenotypic studies have shown that HSTCL cells are positive for CD3 and CD7, and negative for CD5 and B-cell antigens [337, 338]. Most cases of HSTCL are CD4-, CD8-, but a subset may be CD8+. CD56 is expressed in two-thirds of cases, and CD57 is usually negative. The neoplastic cells are positive for TIA-1 and granzyme M, but are usually negative for perforin, and granzyme B, supporting a mature, non-activated cytotoxic T-cell immunophenotype. Most cases of HSTCL are derived from γδ T-cells, hence the original name *hepatosplenic γδ lymphoma*. However, a subset of

cases, approximately 20%, can express the TCR αβ [338, 343], considered a variant of this disease. EBV is usually negative in these tumors.

Conventional cytogenetic analysis of HSTCL has shown a characteristic abnormality, isochromosome 7q [337, 338, 344]. Trisomy 8 is present in a subset of cases. In one study, isochromosome 7q and trisomy 8 were identified in approximately 42% and 33% of cases at the time of diagnosis, respectively, by conventional cytogenetic analysis and/or FISH [338]. The *TRG* and *TRI* genes in HSTCL are usually rearranged. The *TRB* gene also may be rearranged. Recently, mutations of *STAT5B* and *STAT3* were found in a subset of HSTCL cases [345].

Peripheral T-Cell Lymphoma, Not Otherwise Specified

The term peripheral T-cell lymphoma is used to describe lymphoid neoplasms of mature T-cell lineage (as opposed to tumors of thymic origin). The terms post-thymic and mature T-cell lymphoma also have been used to describe these tumors. Peripheral T-cell lymphoma not otherwise specified (henceforth abbreviated as PTCL) is, in part, a diagnosis of exclusion: a lymphoma of mature T-cell lineage that does not fit into other, more specific categories [346]. In an international collaborative study, PTCL represented approximately 15% of all NHLs worldwide, and 25% of all T-cell NHLs [347].

Most patients with PTCL are adults, with a median age ranging from 51 to 68 years in various studies, with a male predominance [346–348]. Patients usually present with lymphadenopathy but extranodal sites of disease are common including skin, liver, Waldeyer ring, and lung. Most patients have advanced stage disease and approximately 50% have B symptoms. A subset of patients can present with eosinophilia, pruritis or hemophagocytic syndrome [349]. Older age, performance status, high serum LDH, and bone marrow involvement are associated with poorer prognosis and have been proposed as components in a prognostic model [348]. Patients with PTCL have a poor overall prognosis; the 5-year survival in different studies has ranged from 25 to 50% [346, 347].

Histologically, in most cases of PTCL the lymph node architecture is diffusely effaced [346, 350]. Vascular proliferation (epithelioid venules) is common and often many inflammatory cells are admixed within the tumor. Cytologically, the neoplastic cells of PTCL exhibit a spectrum of cell sizes including small, medium-sized, and large and can have abundant clear cytoplasm. Anaplastic or Reed-Sternberg-like cells may be found. Mitotic figures are usually easily identified and are often numerous.

There are unusual morphologic variants of PTCL. A subset of these neoplasms can involve the lymph node in paracortical distribution, sparing lymphoid follicles, known as the T-zone variant [346]. Another subgroup has numerous epithelioid histiocytes often arranged in rows, known as the lymphoepithelioid variant of Lennert lymphoma [351]. Rare cases of PTCL can preferentially surround lymphoid follicles in a marginal zone distribution mimicking marginal zone lymphoma, and others preferentially involve the lymphoid follicles mimicking follicular lymphoma [352]. However, in the current WHO classification, follicular variant of PTCL that manifest a T follicular helper (TFH) phenotype is classified as a distinct entity, designated as *Follicular T-cell lymphoma,* which will be discussed later [17].

Immunophenotypic studies have shown that PTCLs are of mature T-cell lineage. Thus, the neoplastic cells express pan-T-cell antigens, usually TCR αβ, and most cases are CD4+, CD8-, (T-helper cell) or CD4- [346, 347]. Approximately 75% of cases of PTCL exhibit an aberrant T-cell immunophenotype which is useful for diagnosis. Cytotoxic proteins, SYK, and CD56/NCAM are expressed by subsets of cases. Attempts have been made to use the immunophenotype to subdivide cases of PTCL: CD4 versus CD8; cytotoxic versus non-cytotoxic, and Th1 (e.g., CXCR3, CD69, CD134, and T-bet) versus Th2 (CD30 and CXCR4). The cytotoxic or Th1 immunophenotypes have been suggested to correlate with worse prognosis [353]. Rare cases of PTCL have been reported to express B-cell antigens, either CD20 or CD79A, but most B-cell antigens and Ig are negative [346]. Proliferation is usually high and Ki-67 rates exceeding 80% are associated with a worse prognosis [347].

Conventional cytogenetic analysis of PTCL cases commonly shows an abnormal karyotype, with a high frequency of complex karyotypes, particularly in tumors composed of large cells [354]. The most common abnormalities, in general, are copy number changes, but structural rearrangements also occur. There are no consistent abnormalities in PTCL. Comparative genomic hybridization analysis of PTCLs has shown a number of gains and losses [355, 356]. Different studies show different regions of gain or loss. In a study by Nelson and colleagues, the most frequent chromosomal gains were 1q, 3p, 5p, 7q (possibly *CDK6*), and 8q24 (including *MYC*), and the most frequent sites of loss were 6q and 10p [356]. Single nucleotide polymorphism (SNP) analysis also has shown many recurrent gains and losses, with gains of 2p15-16/*REL* in approximately one quarter of cases assessed [357]. In these cases, NF-κB pathway activation is documented [357].

PTCLs show monoclonal TCR gene rearrangements [346]. The IG are usually in the germline configuration. The T-cell receptor gene loci are uncommonly involved in chromosomal translocations. Gene expression profiling of PTCL cases has

shown marked heterogeneity, not surprising based on the disease definition [358]. Genes involved in proliferation, apoptosis, matrix remodeling, and signal transduction are overexpressed in subsets of cases [358]. In one study, a subset of cases had a cytotoxic T-cell gene signature that correlated with poorer prognosis [358]. Two major subgroups of PTCL have been suggested, based on overexpression of *GATA3* or *TBX21/Tbet*, with poorer or better prognosis, respectively [359]. Another important finding of gene expression profiling studies is that there is some overlap between PTCL and angioimmunoblastic T-cell lymphoma (AITL) as well as PTCL and ALK-negative anaplastic large cell lymphoma (ALK-ALCL) [358]. These data suggest that the PTCL-NOS category includes subsets of cases of AITL and ALK- ALCL (by gene expression) that lack characteristic histologic or immunophenotypic features. A recent study with whole-exome sequencing identified a highly prevalent *RHOA* mutation encoding a p.Gly17Val alteration present in 18% of PTCL [360].

Follicular T-Cell Lymphoma

A prominent follicular growth pattern can be observed in rare cases of T-cell lymphoma. There is growing evidence that this lymphoma may be a unique clinicopathologic entity based on its morphologic features and derivation from follicular helper T-cells, although this lymphoma also shows overlapping clinical and/or morphologic features with AITL [361, 362]. Therefore, in the current WHO classification, follicular T-cell lymphoma (FTCL) is listed as a provisional entity, and unified under a common heading with AITL [17]. Recurrent mutations of *TET2, DNMT3A,* and *RHOA* that affect a significant proportion of cases of AITL are also observed in FTCL [363–365]. Patients with FTCL more often present with localized disease. Interestingly, mutations of *IDH2*, common recurrent mutations in AITL, are not observed in FTCL [364]. t(5;9)(q33; q22) which involves the *ITK* and *SYK* genes is reported in both FTCL and AITL [366, 367].

Angioimmunoblastic T-Cell Lymphoma

Angioimmunoblastic T-cell lymphoma (AITL) is one of the most common specific types of T-cell lymphoma in Western nations, representing 15–20% of all cases of PTCL [368].

Clinically, patients with AITL are elderly, with a median age in the seventh decade, and no sex preference [368, 369]. AITL is commonly a systemic disease at its onset characterized by advanced stage disease, immunodysregulation, and immunodeficiency. Many patients have constitutional symptoms such as fever, chills, night sweats, malaise, and arthralgias. Patients usually have peripheral lymphadenopathy, often generalized, and extranodal sites of disease are common including

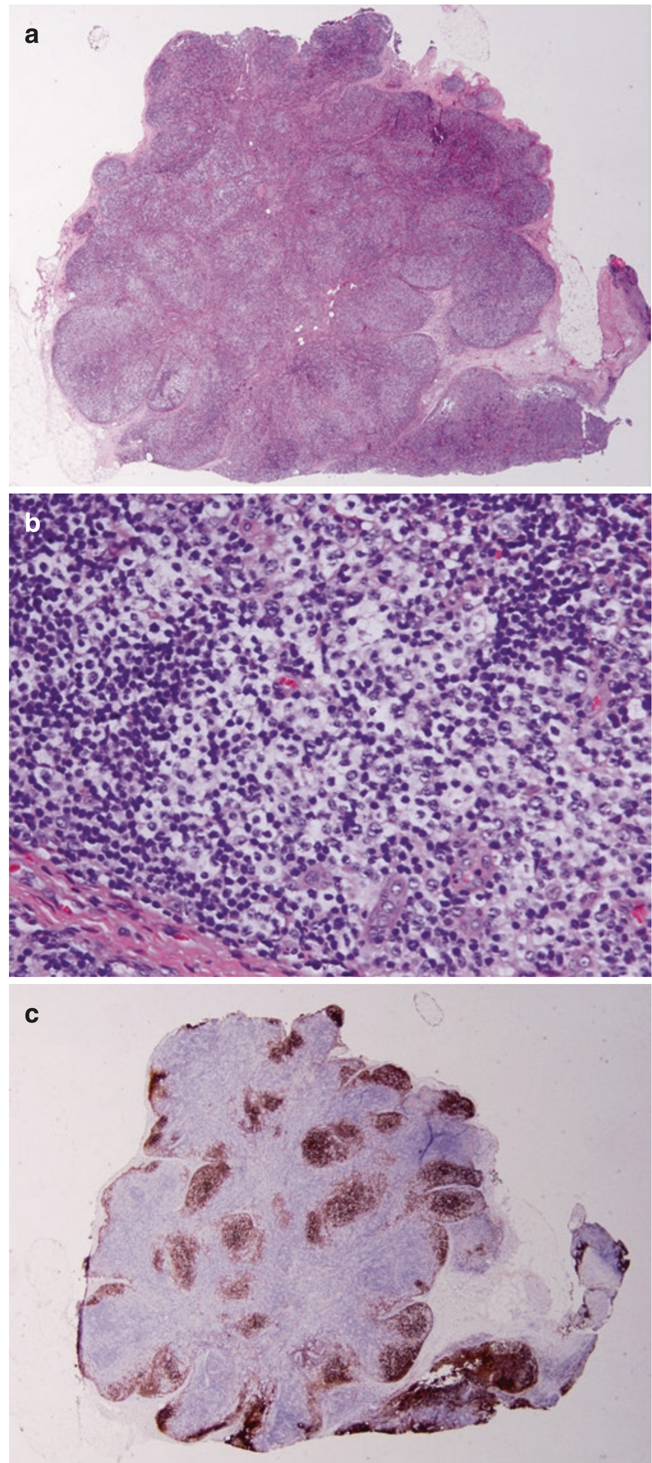

Fig. 40.10 Angioimmunoblastic T-cell lymphoma (AITL) involving lymph node. (**a**) At low magnification the normal nodal architecture can be seen to be replaced. (**b**) Many neoplastic cells with clear cytoplasm are present. (**c**) Immunostaining for CD21 shows many disordered aggregates of CD21+ follicular dendritic cells characteristic of AITL ((**a**) hematoxylin-eosin, 20×; (**b**) hematoxylin-eosin 400×; (**c**) immunohistochemistry with hematoxylin counterstain, 20×)

involvement of liver, spleen, skin, lungs, and bone marrow. Various laboratory abnormalities are common including polyclonal hypergammaglobulinemia, anemia (often hemolytic with positive direct Coombs test), cold agglutinins, circulating immune complexes, cryoglobulins, antinuclear antibodies, eosinophilia, and elevated serum levels of LDH and beta-2-microglobulin. Bone marrow involvement is common. The median survival for all patients with AITL is <3 years, but a subset of patients, approximately 25%, have a more indolent course with better long-term survival [368]. Infectious complications are a common cause of death. Immunomodulator and/or biologic therapeutic agents have been given to patients relatively recently with some promising results, including anti-VEGF (bevacizumab), thalidomide, and cyclosporine A [370, 371]. Age > 60 years, performance status >2, and the presentation with extranodal sites >1, B symptoms and platelet count $<150 \times 10^9$/L were reported to be poor prognostic factors [372].

In lymph nodes AITL can show three general, often overlapping patterns designated as types I, II, and III [368, 373]. In the type I pattern, the overall architecture is partially preserved and many hyperplastic lymphoid follicles are present. In types II and III, there is progressive or complete replacement, respectively, of the lymph node architecture, and small and atrophic (so-called burned-out) germinal centers can be present (Fig. 40.10). A mixture of patterns can occur at presentation, or patients may relapse with a different pattern [373]. The cytological features of AITL are broad, as there is usually an abundant and variable inflammatory infiltrate associated with the neoplastic cells [368, 373]. In fact, in many cases of AITL the neoplastic cells are a minority component in the biopsy specimen. As a result, the histologic appearance of AITL is polymorphous composed of neoplastic small and medium-sized lymphoid cells, often with clear cytoplasm, associated with reactive plasma cells, eosinophils, histiocytes, and B-immunoblasts. Some cases of AITL can have numerous epithelioid histiocytes (mimicking lymphoepithelioid variant of PTCL), as well as plasma cells. These plasma cells can be cytologically atypical and show monoclonal *IGH* rearrangement [374]. Arborizing small blood vessels corresponding to epithelioid venules are present and usually numerous in AITL.

Immunophenotypic studies of AITL have shown that these tumors are of mature T-cell lineage, expressing a variety of pan-T-cell antigens and negative for Ig and B-cell antigens [368]. In addition, most cases of AITL have an immunophenotype closely akin to that of normal follicular T-helper cells [375]. Therefore, the neoplastic cells are CD4+, CD8-, and are often positive for CXCL13, CD10, Bcl-6, PD-1, or CXCR5, as well as other antigens [368, 376]. Normal follicular T-helper cells are located in the light zones of germinal centers and are involved in normal germinal center B-cell differentiation. Their neoplastic transformation in AITL, or the interplay between the neoplastic T-cells and the microenvironment, may explain the presence of abundant non-neoplastic cells in biopsy specimens of AITL including the characteristic proliferation of follicular dendritic cells (CD21+, CD23+). The immunodysregulation and immunodeficiency associated with AITL also may explain the common presence of EBV in non-neoplastic B-immunoblasts in many AITL biopsy specimens. EBV load in tissue biopsy specimens correlates with histologic evidence of progression [377].

Conventional cytogenetic studies have shown a wide variety of numerical and structural abnormalities in approximately 75% of cases of AITL. Trisomies of chromosomes 3, 5, 21, and X and loss of 6q are most common [378]. The presence of a complex karyotype correlates with a poorer prognosis. Comparative genomic hybridization studies have further increased the frequency of detection of chromosomal alterations, identified in up to 90% of AITL cases. EBV is commonly associated with AITLs, as shown by a variety of molecular methods, and is monoclonal in a subset of cases [379]. EBV infection is most likely a secondary event in pathogenesis, as we have observed patients with AITL in which the initial biopsy specimen did not have evidence of EBV, but the virus was abundant at the time of relapse. Gene rearrangement studies have demonstrated that most AITL cases have T-cell receptor gene rearrangements. *IGH* is rearranged in 10–20% of cases [368]. Gene expression profiling studies of AITL have further confirmed the concept that AITL is a neoplasm of follicular T-helper cells [380]. In addition, approximately 90% of the gene signature in AITL is contributed by reactive cells in these lesions, particularly B-cells.

Recently, additional discoveries of the genetic basis for AITL were made. Mutations in the epigenetic regulators *TET2*, *DNMT3A*, and *IDH2* frequently occur in AITL, but are not specific [381, 382]. Most specific to AITL is a point mutation in the Ras homolog gene family member A (*RHOA* G17 V) in almost 70% of AITL samples, resulting in a disruption of RHOA signaling [364]. However, this was also present in PTCL with a TFH-immunophenotype. These studies suggest that these mutations may be acquired in a multistep manner, because all the *RHOA*-mutated samples are *TET2* mutated, and the smaller percentage of *IDH2*-mutated tumors harbored both *TET2* and *RHOA* mutations [364].

Patients with AITL are at risk for developing B-cell lymphomas, particularly DLBCL, and less frequently classical Hodgkin lymphoma or plasmacytoma [368, 383]. A subset of cases of DLBCL and most classical Hodgkin lymphoma arising in this setting are EBV+. One possible explanation for this phenomenon is that EBV infection prolongs B-cell lifespan, increasing the likelihood of secondary molecular aberrations that result in lymphoma. In some patients, the diagnosis of DLBCL can precede the diagnosis of AITL.

Anaplastic Large Cell Lymphoma, ALK-Positive

In 1985, Stein and colleagues [384] identified a subset of NHLs characterized by anaplastic large lymphoid cells, and originally named Ki-1 lymphoma. This neoplasm was subsequently designated as anaplastic large cell lymphoma (ALCL). In the current WHO classification, there are four categories of disease that include the term ALCL: ALK+ ALCL and ALK-ALCL which are their own entities, Breast implant-associated ALCL which is currently considered a provisional entity, and cutaneous ALCL, the latter being a well-established, separate category of disease [17]. ALK+ ALCL is defined as a T-cell lymphoma, usually composed of anaplastic cells, associated with molecular alterations of the *ALK* gene, ALK overexpression, and uniform expression of CD30 [385].

ALK+ ALCL affects children and adults, with a wide age range, but is most common in the first three decades of life [385–387]. There is a male predominance, 1.5 to 1. B symptoms are common in ALK+ ALCL patients, particularly high fever, and approximately 75% of patients have advanced stage disease. Many patients have a high IPI and serum levels of LDH or beta-2 microglobulin are often high. Lymph nodes are the most common site of involvement, but extranodal sites are commonly involved, most often skin, soft tissue, bones, and lungs. Bone marrow is involved in approximately 20% of patients with ALK+ ALCL if searched for diligently using immunohistochemical analysis. Leukemic involvement is uncommon in patients with ALK+ ALCL, but very high leukocyte counts can occur. ALK+ ALCL is clinically aggressive and patients require combination chemotherapy, however, prognosis is favorable. The overall 5-year survival ranges from 60 to 80%.

Histologically, ALK+ ALCL can partially or completely replace lymph node architecture [385–387]. In partially involved lymph nodes, the tumor tends to infiltrate sinuses, or preferentially replaces the paracortex (T-cell region). Necrosis is common and there are high numbers of apoptotic cells and mitotic figures. A starry-sky pattern occurs in a subset of cases. Cytologically, ALK+ ALCL can exhibit a remarkably wide spectrum. The WHO classification recognizes five variants: common or typical (60%) (Fig. 40.11), lymphohistiocytic (10%), small cell (5–10%), Hodgkin-like (3%), and composite or mixed (15%) [385]. The lymphohistiocytic variant is composed of relatively few neoplastic cells associated with numerous lymphocytes and histiocytes. In the small cell variant, large anaplastic neoplastic cells are infrequent and many small lymphoma cells are present. Small cell and lymphohistiocytic components are reported to be associated with poorer response to therapy in the pediatric population [388]. There are also other morphologic variants that are less common including: monomorphic (resembling DLBCL or plasmacytoma), sarcomatoid, eosinophil-rich, neutrophil-rich, and myxoid. In most

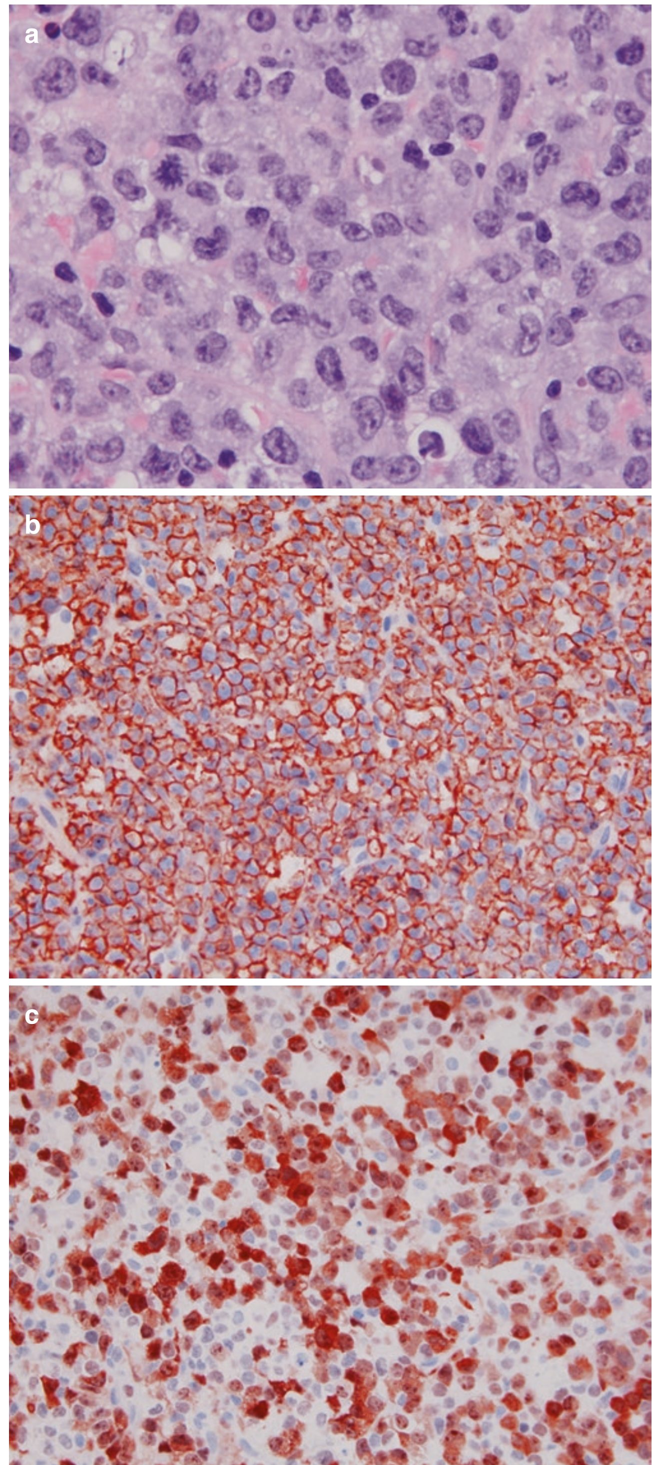

Fig. 40.11 Anaplastic lymphoma kinase (ALK) + anaplastic large cell lymphoma involving lymph node. (**a**) The lymphoma cells are large and a subset has horseshoe-shaped nuclei consistent with so-called hallmark cells. (**b**) The lymphoma cells uniformly and strongly express CD30. (**c**) The lymphoma cells express ALK in a nuclear and cytoplasmic pattern consistent with t(2;5)(p23;q35)/NPM-ALK. ((**a**) hematoxylin-eosin, 1000×; (**b**, **c**) immunohistochemistry with hematoxylin counterstain, 400×)

cases of ALK+ ALCL, irrespective of the variant, at least some hallmark cells are present. These are large cells with a horseshoe- or kidney-shaped nucleus with vesicular chromatin and prominent nucleoli, abundant and basophilic cytoplasm, and a paranuclear clear area or hof [385].

Immunophenotypic studies have shown that all cases of ALK+ ALCL express CD30, with a membranous and paranuclear pattern, as well as ALK [385–387]. Virtually all cases of ALK+ ALCL strongly express CD25, and most cases express EMA/MUC1, T-cell antigens (most often CD2, CD4, CD5, CD43, or CD45RO), cytotoxic antigens (granzyme B and TIA-1), and CD45/LCA (often dim and best detected by flow cytometry). Other molecules often expressed in ALK+ ALCL include: AP-1 transcription factors, phosphorylated (active) STAT3, Bcl-3, cyclin D3, clusterin, fascin, serpin A1, MCL-1, and BAX [368, 389–391]. P53 is commonly expressed but rarely mutated [392]. Ki-67 immunostaining shows a variable but usually high proliferation rate. Bcl-2 is virtually always absent, as is EBV (either EBER or LMP1) and CD117/KIT, and CD15 is very rarely expressed [385, 393]. ALK+ ALCLs are negative for Ig and B-cell antigens. Approximately 10–20% of cases of ALK+ ALCL are immunophenotypically "null cell" without any T-cell antigens being expressed. This occurs more often when only immunohistochemical analysis is used, as a lesser number of antibodies is available, and this method is less sensitive than flow cytometry.

Conventional cytogenetics studies initially showed the t(2;5)(p23;q35) translocation in a subset of cases initially designated as malignant histiocytosis and subsequently as ALCL [394]. The breakpoints of this translocation were then cloned, showing *ALK* at chromosome 2p23 and the nucleophosmin (*NPM1*) at chromosome 5q35 [395]. These genes are disrupted to create a novel *NPM1-ALK* fusion gene [395]. There are many additional molecular alterations of *ALK* including reciprocal translocations and one inversion [387]. These *ALK* gene abnormalities are now known to be characteristic of ALK+ ALCL and are included in the definition of this neoplasm. The t(2;5)(p23;q35) is most common, identified in approximately 80% of cases of ALK+ ALCL. The t(1;2)(q25;p23) involving *TPM3* occurs in approximately 10% of ALK+ ALCLs, and all other known translocations are uncommon, in the range of <1 to approximately 2% [385]. A study by Feldman and colleagues identified a novel *TRAF1-ALK* fusion using deep RNA sequencing [396]. The discovery of this *TRAF1-ALK* fusion expands the diversity of known ALK fusion partners and highlights the power of deep sequencing for fusion transcript discovery [396].

The pattern of ALK expression, as shown by immunohistochemical analysis, correlates with the molecular abnormalities present [385, 387]. Tumors carrying the t(2;5) have a nuclear and cytoplasmic pattern of ALK expression (Fig. 40.11c). This is because NPM1 can migrate from the cytoplasm into the nucleus. The rare t(2;X)(p23;q11–12) involves *moesin* (*MSN*). Tumors with this translocation have a membranous pattern of ALK expression, because *MSN* is located in the membrane. All other translocations result in a cytoplasmic pattern of ALK expression. The t(2;17)(p23;q23) involving *clathrin* (*CLTC*) is distinctive because the ALK expression pattern appears flocculent. This is true because CLTC is normally located in the membranes of cytoplasmic vesicles.

ALK+ ALCLs usually carry TCR gene rearrangements. Most of the "null cell' cases also carry TCR gene rearrangements but approximately 10% do not. Perhaps this subset of tumors arises from an early T-cell progenitor, at a stage of differentiation prior to gene rearrangement. The *perforin* gene is reported to be mutated in a subset of cases [397]. The *SHP1* and *RB* genes are commonly inactivated or deleted [398]. Comparative genomic hybridization analysis has shown areas of chromosomal gain or loss in approximately 60% of ALK+ ALCLs, including gains of chromosome 7, 17p, or 17q24, and losses of 4q13–21, 11q14, or 13q [399]. Gene expression profiling has shown that ALK+ ALCL has a different signature than ALK- ALCL, with overexpression of over 100 genes [400]. The top four genes are *BCL6*, *PTPN12*, *CEBPbeta*, and *serpin A1*. A number of studies have elucidated pathways that are active in ALK+ ALCL including pathways involved globally in proliferation, ribosome synthesis, survival, apoptosis evasion, angiogenesis, and cytoarchitectural organization.

The NPM-ALK fusion protein activates various signaling pathways in ALK+ ALCL cells, including the JAK/STAT3, PI3K/AKT/mTOR, RAS/ERK, and PLCγ pathways. Rearrangements of *MYC* have been reported in ALK+ ALCL. All these patients with dual *ALK* and *MYC* rearrangements demonstrated an aggressive clinical course, with systemic and extranodal presentation, early tumor relapse, and bone marrow involvement during the course of the disease [401, 402].

Anaplastic Large Cell Lymphoma, ALK-Negative

This disease is defined in the WHO classification as a CD30+ T-cell lymphoma "not reproducibly distinguishable on morphological grounds from ALK-positive ALCL, but lacks ALK protein" [403]. Recent clinical, pathologic, and genetic data have revealed significant heterogeneity in ALK- ALCL [404].

Patients with ALK- ALCL can be any age, but most patients are adults 40–65 years old. The male-to-female ratio is 1.5 to 1 [403]. Lymph nodes are most commonly involved but extranodal sites can be involved, most often skin, soft tissues, and bones. Patients often have B symptoms, advanced stage disease, and an aggressive clinical course requiring combination chemotherapy [403].

Histologically, most cases of ALK- ALCL resemble the common variant of ALK+ ALCL [386, 403, 404]. The neoplasm has a tendency to infiltrate lymph node sinuses. The lymphoma cells are large and often more pleomorphic, with a higher nucleus-to-cytoplasm ratio than observed in ALK+ ALCL. Hallmark cells, however, are present. Immunophenotypic studies show uniform expression of CD30 in a membranous and paranuclear pattern and absence of ALK [403]. ALK- ALCLs express T-cell antigens and most cases are positive for cytotoxic molecules and clusterin [386, 387, 403]. Many cases of ALK- ALCL express Bcl-2 and approximately 50% of cases express survivin. A subset of cases can express EMA or CD15, and all cases are negative for Ig and B-cell antigens.

Until recently, the genetics of ALK- ALCL has been unknown. A recent study by Parrilla and colleagues identified *DUSP22* and *TP63* rearrangements in 30% and 8% of systemic ALK-negative ALCLs, respectively [404]. The former involves the *DUSP22-IRF4* locus on 6p25.3, and is most commonly present as t(6;7)(p25.3;q32.3) [405]. *DUSP22* is a dual-specificity phosphatase that inhibits T-cell receptor signaling by inactivating MAP/ERK [406]. Most frequent partner gene of *TP63* is *TBL1XR1* [407]. *TP63* encodes the p53 family member, p63, and the gene rearrangements encode p63 fusion proteins which have putative oncogenic function [407]. These rearrangements are mutually exclusive, and are uniformly absent in ALK+ ALCL. *DUSP22* rearranged cases have favorable outcomes similar to ALK+ ALCL. *TP63* rearranged ALCLs have a poor prognosis.

Breast Implant-Associated Anaplastic Large Cell Lymphoma

Breast implant-associated ALCL is included as a provisional entity in current WHO classification [17]. Both saline and silicone filled implants have been implicated, with a median interval from the time of the implant to the lymphoma of about 10 years [408]. Histologically, breast implant-associated ALCLs are composed of large pleomorphic cells that are usually confined to serous fluid or the capsule surrounding the implant [408–410]. Immunophenotypic studies have shown strong and uniform expression of CD30 and T-cell antigens, with absence of ALK in all cases [410]. Many breast implant-associated ALCLs also express cytotoxic proteins, clusterin, and EMA. These neoplasms carry monoclonal TCR gene rearrangements.

In most cases the neoplastic cells are confined to the seroma fluid, without invasion of the capsule. In such cases conservative management is recommended, with removal of the implant and capsule [411]. If there is invasion through the capsule, there is a risk of lymph node involvement and systemic spread, warranting systemic chemotherapy [411]. The risk factors to develop this lymphoma have not yet been established.

Hodgkin Lymphoma

Hodgkin lymphoma (HL) represents approximately 10–15% of all lymphomas. The older classification of Jackson and Parker [412] divided HL into three groups: paragranuloma, granuloma, and sarcoma (Table 40.4). Although this classification recognized the excellent prognosis of patients with paragranuloma and the poor prognosis of patients with sarcoma, most cases fit within the granuloma group. This category was very heterogeneous in terms of clinical presentation, histologic findings, and prognosis thereby limiting the utility of the Jackson and Parker system. In 1966, Lukes and Butler [413] developed another HL classification in which six categories were recognized: lymphocytic and/or histiocytic (L&H) nodular, L&H diffuse, nodular sclerosis, mixed, diffuse fibrosis, and reticular. These categories recognized the distinctive neoplastic cells and the composition of the reactive cells found in the different types of HL. Subsequently, at an international symposium in Rye, New York, the Lukes and Butler classification was consolidated into four groups: lymphocytic predominance, nodular sclerosis, mixed cellularity, and lymphocytic depletion

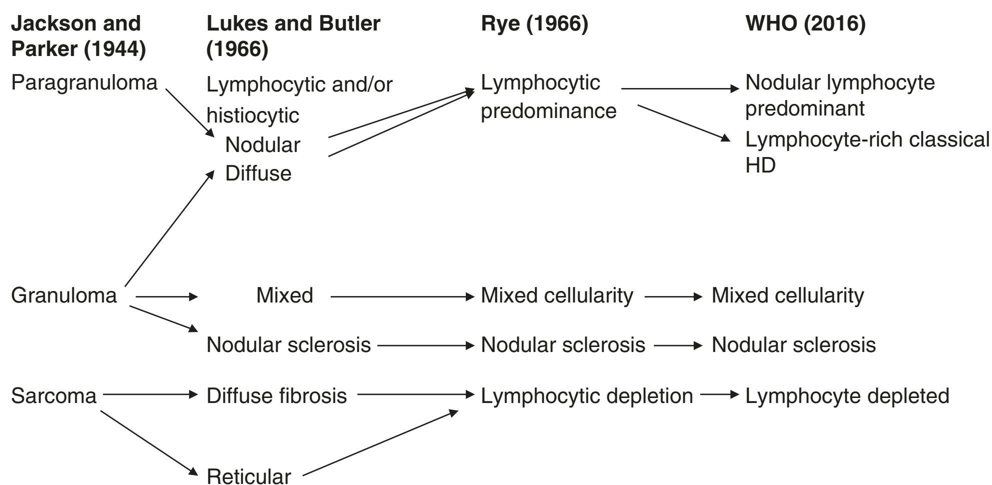

Table 40.4 Hodgkin lymphoma classification: historical and present

[414]. The L&H nodular and diffuse groups were combined into the lymphocytic predominance category and the diffuse fibrosis and reticular groups were consolidated into the lymphocytic depletion category. The Rye classification gained universal acceptance, was easy to use, and the categories correlated with clinical parameters. Nevertheless, some useful information was lost in the consolidation of the Lukes and Butler classification. This information was subsequently recaptured and in the 1980s and 1990s. In the WHO classification, two broad categories of HL are recognized: nodular lymphocyte predominant and classical HL. The latter group is composed of four types: nodular sclerosis, mixed cellularity, lymphocyte-rich classical, and lymphocyte depleted [17].

Hodgkin lymphomas share a number of clinical and pathologic features that distinguish them from NHLs. Hodgkin lymphomas tend to occur in children and younger adults, and arise in lymph nodes. Extranodal sites of origin are rare. Classical HLs begin in the interfollicular region of lymph node and progressively efface the architecture of that lymph node, and then usually spread in an orderly fashion to contiguous lymph node groups. This pattern of spread is quite different from many types of NHL. Another distinctive feature is the cellular composition of HLs. The neoplastic cells in a biopsy specimen involved by HL are usually only a minor component, often less than 1% of all cells in the specimen. The neoplastic cells are known as lymphocyte predominant (LP) cells in nodular lymphocyte predominant HL and as Reed-Sternberg and Hodgkin (RS + H) cells in the classical HL types. The remaining cells in biopsy specimens are reactive inflammatory cells.

Approximately 75% of HL patients can be cured with appropriate therapy and all histologic types are responsive to standard therapy. The major findings that correlate with response to therapy include patient age, presence or absence of systemic symptoms, stage, tumor bulk, and laboratory findings such as a high serum LDH level [415]. Although histologic type does not correlate with response to therapy, classification remains useful. In nodular lymphocyte predominant, the LP cells strongly express CD20, and rituximab has been used in therapeutic protocols [416]. For classical HL, brentuximab vedotin (antitubulin agent monomethyl auristatin E conjugated to CD30-specific monoclonal antibody) induces durable responses for relapsed or refractory cases [417, 418]. The HL type also correlates, in part, with patient age, sites of involvement, stage at presentation, and the presence of systemic symptoms [419].

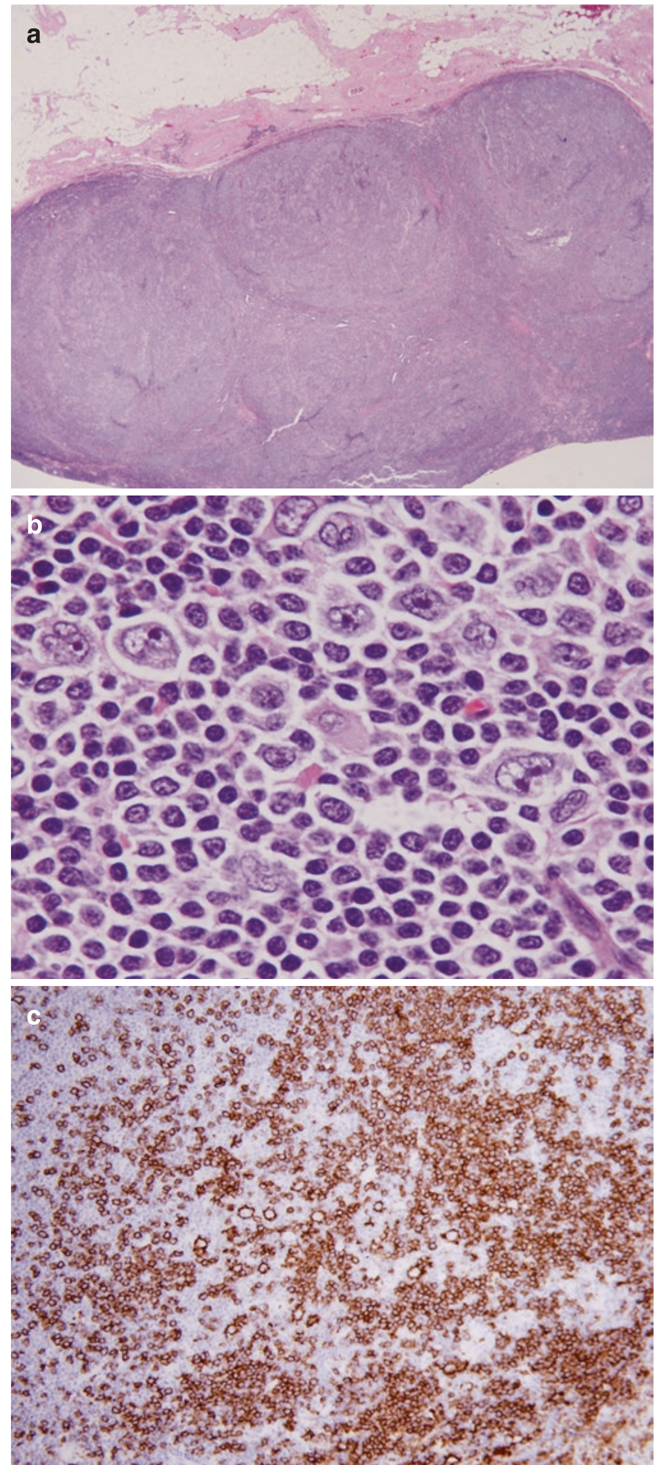

Fig. 40.12 Nodular lymphocyte predominant Hodgkin lymphoma involving lymph node. (**a**) Nodular pattern is obvious at low magnification. (**b**) High magnification of one nodule shows many large neoplastic lymphocyte predominant (LP) cells in a background of reactive small lymphocytes and histiocytes. (**c**) Immunostaining for CD20 shows that LP cells and many small reactive lymphocytes are CD20+ B-cells ((**a**, **b**) Hematoxylin-eosin, (**a**) 20× and (**b**) 1000×; (**c**) immunohistochemistry with hematoxylin counterstain, 200×)

Nodular Lymphocyte Predominant HL

Approximately 5% of patients with HL have the nodular lymphocyte predominant (NLP) type, which is usually localized and most involves cervical or axillary lymph nodes [420, 421].

Spleen, liver, and bone marrow are rarely involved (unless transformation to DLBCL has occurred). The disease can affect patients of all ages, but most patients are younger adults, between 30 and 50 years of age. There is a marked male predominance, with a male-to-female ratio up to 3 to 1 in some studies. Systemic symptoms, such as fever, weight loss, and night sweats are infrequent. This disease is clinically indolent. In an epidemiologic study from the NCI Statistics, Epidemiology, and End Results (SEER) program, the 5-year overall survival rate of patients with NLPHL was 83.9% [422].

Histologically, NLPHL is characterized by effacement of nodal architecture by variably sized, vague nodules composed of numerous small lymphocytes, histiocytes, and characteristic neoplastic LP (previously known as L&H) cells (Fig. 40.12) [420]. These cells are large with pale cytoplasm and polyploid, vesicular nuclei containing inconspicuous, basophilic nucleoli. LP cells resemble kernels of popped corn, hence the nickname *popcorn cells*. Eosinophils, neutrophils, and plasma cells are usually absent. Cells resembling Reed-Sternberg cells are rare. There is no associated necrosis or sclerosis, but sclerosis can be present at the time of relapse. It has long been recognized that NLPHL can have varied growth patterns, including some with diffuse areas and/or numerous T-cells. Fan and colleagues designated six histologic patterns; "classic" (B-cell-rich) nodular, serpiginous/interconnected nodular, nodular with prominent extranodular LP cells, T-cell-rich nodular, T-cell-rich diffuse large B-cell like, and a (diffuse) B-cell-rich pattern [423]. Among these, the typical histopathologic growth patterns in NLPHL include "classic" (B-cell-rich) nodular, and serpiginous/interconnected nodular subtype, and these comprise 75% of NLPHL cases. Twenty-five percent of NLPHL cases show one of the variant patterns, and these have been reported to be associated with advanced disease and a higher relapse rate [424].

Immunohistochemical studies have shown that NLPHL is distinct from other types of HL [420]. The LP cells express CD45, Ig J chain, pan-B-cell antigens (bright expression), and Bcl-6. The LP cells can express EMA or IgD in approximately 50% and 25% of all cases, respectively, and LP cells are almost always negative for CD15 and CD30. The neoplastic nodules can be observed to be centered in follicles. The nodules have a prominent network of follicular dendritic cells (CD21+, CD23+, and/or CD35+) and many intrafollicular T-cells (follicular helper T-cells) are present that often express CD4, CD10, CD57, Bcl-6, and PD-1. These T-cells can surround and form rosettes around the LP cells. The combination of histologic and immunophenotypic findings also allows one to appreciate the spectrum of patterns in cases of NLPHL.

Single cell PCR analysis of LP cells in NLPHL have shown monoclonal *IGH* gene rearrangements and a high number of somatic mutations in the variable regions, with evidence of ongoing mutations, consistent with a germinal center-derived B-cell tumor [425, 426]. These rearrangements are usually functional and Ig mRNA transcripts can be identified in most LP cells. Mutations of B-cell genes (e.g., *PAX5*) have been reported in a subset of cases and approximately 50% of tumors carry *BCL6* rearrangements [427]. Comparative genomic hybridization analysis of NLPHL revealed a high number of genomic imbalances in LP cells [209]. The chromosomes most often involved in that study were gains of chromosomes 1, 2q, 3, 4q, 5q, 6, 8q, 11q, 12q, and X, and loss of chromosome 17. A gene expression profiling study of microdissected LP cells showed that these cells have a gene signature corresponding to a transitional stage between germinal center B-cells and memory B-cells. LP cells further show a partial loss or defective B-cell phenotype with upregulation of genes in the NF-κB pathway and dysregulation of apoptosis [428]. Both NLPHL and CHL are characterized by constitutive activity of the NF-κB pathway, however, the mechanisms of underlying activation of NF-κB pathway are different between these two [428, 429]. The inactivating mutations found in genes such as *NFKBIA* and *TNFAIP3* in CHL are not present in most cases of NLPHL [429]. EBV infection, which is known to drive NF-κB activation in some cases of CHL, is rare in NLPHL.

Classical Hodgkin Lymphoma

Lymphocyte-Rich Classical HL

In the past decade, cases of classical HL were recognized that histologically resemble NLPHL, in either a nodular or diffuse pattern, but immunophenotypically resemble the classical types of HL [430]. In the WHO classification, these neoplasms are designated as *lymphocyte-rich classical HL* [431]. Others have questioned whether lymphocyte-rich classical (LRC) HL should be designated as a distinct type, or is more simply an early or transitional form of either nodular sclerosis or mixed cellularity HL.

In the largest study of LRCHL, including 145 patients as part of the German Hodgkin's Study Group, LRCHL represented 5% of all cases of HL [432]. The median age of affected patients is 38 years and there is a male predominance. B symptoms are uncommon and patients usually present with either stage I or II disease, most often involving cervical, supraclavicular, or axillary lymph nodes. The prognosis of patients with LRCHL is good, similar to patients with NLPHL [432].

The neoplastic RS + H cells in LRCHL have the immunophenotype of classical HL, being positive for CD15, CD30, PAX5 (dim), and negative for CD45/LCA [431]. In addition, the neoplastic cells seem to have intermediate features

between the LP cells of NLPHL and RS + H cells of classical HL [433]. In LRCHL the neoplastic cells more frequently express Bcl-6 and B-cell transcription factors than the RS + H cells of classical HL [433]. The background inflammatory cells in LRCHL are closer to that of follicles, more similar to NLPHL than classical HL, and lack eosinophils and neutrophils. In addition, a follicular T-cell microenvironment was identified in 50% of lymphocyte-rich classical Hodgkin's lymphoma cases [433]. Following these findings, LRCHL appears to occupy an intermediate position between classical HL and NLPHL, nevertheless, molecular studies of the neoplastic cells in LRCHL have shown *IGH* gene rearrangements with variable region mutations, without evidence of ongoing mutations, more in keeping with classical HL [434].

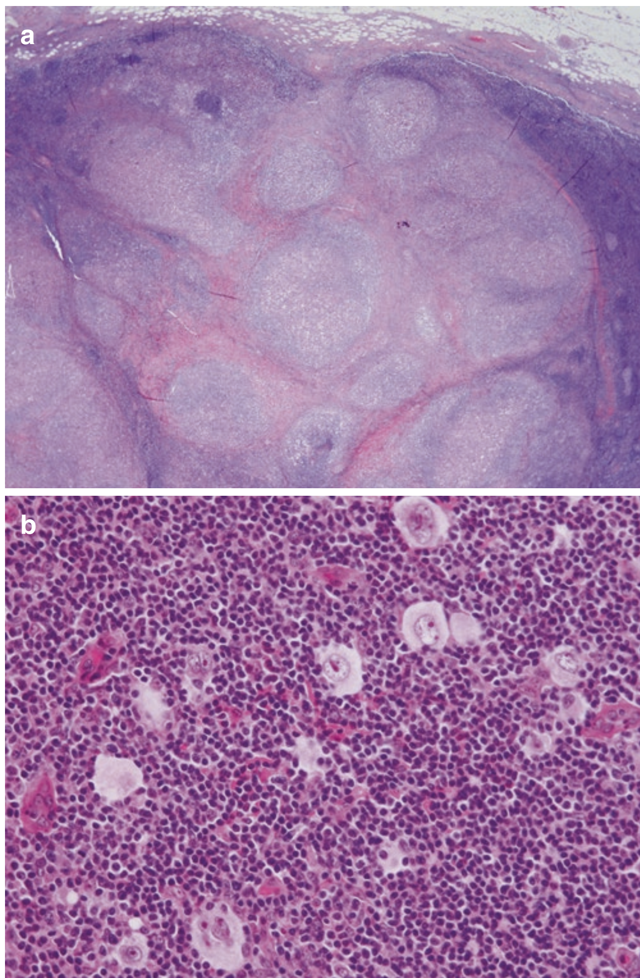

Fig. 40.13 Nodular sclerosis Hodgkin lymphoma. (**a**) Low magnification shows nodules of tumor surrounded by dense fibrous (sclerotic) bands. (**b**) Large, neoplastic lacunar cells in a background of predominantly small reactive lymphocytes (hematoxylin-eosin, (**a**) 20×; (**b**) 400×)

Nodular Sclerosis HL

Nodular sclerosis (NS) is the most common form of HL, representing approximately 70% of all cases [422, 435]. It is the most common type of HL below the age of 50 years, with the peak age range being between 15 and 35 years. The age-adjusted incidence rate of NS has increased in the United States over the past three decades [422]. This increase was greatest in adolescents and young adults. The disease affects men and women with equal frequency. Whites are affected more often than blacks and Hispanics. There is a correlation between overall economic resources and the frequency of disease, with wealthy countries and individuals of higher socioeconomic status having a higher incidence of NSHL.

NSHL has a marked predilection for involving cervical, supraclavicular, and mediastinal lymph nodes and the thymus can be involved [435]. B symptoms occur in up to 30–40% of patients. Approximately 80% of patients have a mediastinal mass, which often is bulky, and therefore most patients present with stage II disease. The disease can invade into the lungs. The bone marrow is involved in less than 5% of patients at the time of diagnosis. According to an NCI SEER study, the 5-year survival rate was 82.2% [422].

Histologically, NS is characterized by a triad of findings: (1) a nodular pattern, (2) broad bands of fibrosis that surround nodules, and (3) a characteristic mononuclear cell variant known as a lacunar cell (Fig. 40.13) [435]. According to the WHO classification definition of NSHL, at least one nodule must be surrounded by fibrous bands [435]. A lacunar cell has abundant clear cytoplasm with a sharply demarcated cell membrane. In formalin-fixed tissue the cell cytoplasm retracts leaving a clear space or lacuna surrounding the cell, which led to its name. The lacunar cell nucleus may be hyperlobulated or may contain multiple small nucleoli. The nucleoli are usually smaller than those seen in classic RS + H cells. A heterogeneous inflammatory cell background can be seen in NSHL, including small lymphocytes, histiocytes, eosinophils, neutrophils, and plasma cells in a variable mixture. Cases of NSHL with sheets of lacunar cells, often associated with necrosis and lymphocyte depletion, have been designated as syncytial variant of NSHL, and are reported to have a poorer prognosis [436]. Efforts have been made to grade cases of NSHL based on the numbers of neoplastic cells and reactive cells. British National Lymphoma Investigation (BNLI) proposed a grading system to subclassify into NS I and NS II, based on the number of RS + H cells, atypia, and quality and quantity of fibrosis. However, prognostic significance is controversial. Later, The German Hodgkin Lymphoma Study Group (GHSG) proposed a grading system based on the three risk factors (eosinophilia, lymphocyte depletion, and atypia of the RS + H cells). Cases with one or more risk factors are subclassified as high risk, and have poorer prognosis [437].

Mixed Cellularity HL

Mixed cellularity (MC) is the second common type of HL, representing approximately 25% of cases in wealthy countries, and is more frequent in poorer countries and in patients with HIV infection [422, 438]. This type is the most common form of HL in patients older than 40 years. Males are affected more often than females, with a ratio of approximately 2 to 1. In African-Americans and Hispanics, MCHL is relatively more common than in whites. Most patients present with peripheral lymphadenopathy. The mediastinum is rarely involved. B symptoms are common, in approximately 50–60% of patients, and most patients have clinical stage III or IV disease. According to NCI SEER data, the 5-year overall survival rate of patients with MCHL is 68.1% [422].

Histologically, MCHL is characterized by a large number of classic Reed-Sternberg cells and mononuclear (Hodgkin) cell variants. The lymph node architecture is usually diffusely replaced, but partially involved lymph nodes show selective paracortical infiltration. Rarely, a purely interfollicular pattern is present. Focal necrosis may be found, but the necrosis is usually not prominent. The reactive cell component is commonly composed of eosinophils, plasma cells, histiocytes, and small lymphocytes in variable proportions. In some cases histiocytes can predominate, often in clusters and with epithelioid features, making it difficult to identify the neoplastic cells. EBER is more frequently positive (approximately 75% of cases) than in nodular sclerosis and lymphocyte-rich classical HL [438].

Lymphocyte-Depleted HL

Lymphocyte-depleted (LD) is the least common type of HL, representing less than 1% of cases [439]. The median age of patients with LDHL occurs in the fourth or fifth decades, and the male-to-female ratio is approximately 2 to 1. Whites and blacks are equally affected [422]. Patients with LDHL are older, and present more often with advanced disease, and more frequent B symptoms than patients with other subtypes of HL [440, 441]. Patients with LDHL have the poorest prognosis of all types of HL, with a 5-year survival rate of 36.4% in a NCI SEER study [422].

Karube et al. reported that LD type represents an independent prognostic factor among classical Hodgkin lymphoma by multivariate analysis [441].

Grossly, the extent of tumor is often greater in LDHL than in other types of HL, and patients commonly have a large contiguous mass of matted lymph nodes or diffuse visceral involvement. In patients with LDHL, the incidence of non-contiguous lung involvement is high, suggesting vascular spread.

The age-adjusted incidence rate for LDHL in the NCI SEER database has decreased over the past decades [422]. This decrease is most likely explained by the recognition by pathologists that many tumors previously classified as LDHL are, in fact, NHLs [422, 442]. Improved classification is the result of application of immunohistochemical and molecular methods to the study of lymphoid neoplasms.

Histologically, LDHL can be subdivided into diffuse fibrosis and reticular variants as defined by Lukes and Butler. In the diffuse fibrosis variant, the tumor is characterized by an extensive proliferation of disordered, hypocellular fibrosis [439–441]. Reed-Sternberg and Hodgkin cells are usually numerous and may be spindled within dense collagen. Reactive inflammatory cells are relatively few. In the reticular variant, there are numerous RS + H cells and bizarre variants that have been termed pleomorphic variants. These cells may exhibit extreme variations in nuclear number and shape, often with giant nucleoli. Necrosis is common and may be extensive. Mitotic figures are usually numerous. EBV infection is common in LDHL.

Immunophenotypic Findings of Classical HL

In general, analysis of tissues involved by classical HL using flow cytometry is not helpful. The RS + H cells are typically infrequent in involved tissues and thus are difficult to assess [443]. Immunohistochemical methods are more helpful because this technique allows direct observation of RS + H cells as well as reactive cells. Thus, most pathologists currently assess HL cases using fixed tissue sections [443]. Reed-Sternberg and Hodgkin cells express CD15 (60–70%) and CD30 (>95%), and are negative for CD45/LCA. The B-cell differentiation program of RS + H cells is highly defective, and therefore these cells express pan-B-cell antigens such as CD20 variably, and in only a subset of cases, approximately 20%. A number of B-cell transcription factors are either weakly expressed by RS + H cells (e.g., PAX5), or are expressed in only subsets of cases (e.g., OCT2, BOB.1) [443]. Ki-67 immunostaining shows that most RS + H cells are positive and therefore proliferating. Bcl-2 is positive in up to half of classical HLs and has been correlated with poorer prognosis. NF-κB is expressed by RS + H cells in most cases of HL [443]. T-cell antigens can be aberrantly expressed by RS + H cells in approximately 10% of classical HL cases, but almost all of these tumors carry *IGH* rearrangements and are of B-cell origin [444]. The background lymphocytes in classical HL are a mixture of polytypic B-cell and T-cells, and histiocytes are also present. It is clear that the tumor microenvironment is very important. Expression levels of FOXP3, CD68, and CD20 in a background cells are also reported to be associated with prognosis [445].

EBV is present in the RS + H cells of classical HL in over two-thirds of cases of MCHL and LDHL, and in approximately 20% of NS [422, 443, 446]. Most cases of HL arising in the setting of HIV infection are EBV+. When present, EBV is present in monoclonal, episomal form and viral proteins are expressed with a type II latency pattern [446].

Molecular Genetic Findings of Classical HL

Conventional cytogenetic studies have revealed non-random complex abnormalities in up to half of HL cases. Chromosomal breakpoints have been reported to involve 6q15-16, 7q31-35, 8q22-24, 11q32, 12p11-13, 13p11-13, and 14q32 [447]. Comparative genomic hybridization methods also have shown a wide, variable number of chromosomal copy number changes occurring in subset of cases of classical HL [448].

For years the origin of the RS + H cells in classical HL was in doubt. However, with the onset of single-cell PCR methods it is now clear that almost all (if not all) cases of classical HL are of B-cell lineage [425, 426, 443]. The RS + H cells have *IGH* gene rearrangements and variable regions carry a high load of somatic mutations, and sometimes crippling mutations. In addition, a number of proteins are aberrantly expressed in classical HL (e.g., ID2) that, in combination with the molecular findings, may explain the defective B-cell differentiation that is characteristic of RS + H cells. Gene expression profiling and other methods have shown that a number of pathways are important in the pathogenesis of classical HL, with the NF-κB and JAK-STAT pathways being prominently involved, but genes involved in angiogenesis, apoptosis, extracellular matrix remodeling, proliferation, and cytokines also involved [449, 450]. Several studies have gone on to further implicate activation of the NF-κB pathway, via mutations in a number of genes including *NFKBIA, NFKBIE, TNFAIP3, REL*. Of note, these mutations are far less frequent in EBV-positive CHL, suggesting that the virus may replace the role of mutations in NF-κB pathway activation. Hypermethylation, a well-established modality of tumor suppressor gene inactivation, has been shown to be consistently present in the promoter regions of several key B-cell–specific transcription factors such as BOB.1 and PU.1 [451]. Furthermore, aberrant expression of *NOTCH1* and *ID2* involved in T-cell and NK-cell differentiation may cooperate with the aforementioned mechanisms to mask the B-cell gene signature of these cells. In conjunction with downregulation of the B-cell gene signature, several other pathways including the MAP/ERK and JAK/STAT pathways also promote survival and proliferation in CHL [452, 453].

Hodgkin Lymphoma and the Two-Disease Hypothesis

For many years epidemiologists have suggested that HL is heterogeneous and represents more than one disease [454]. This two-disease hypothesis suggested NSHL and MCHL as two different diseases. The epidemiologic, clinical, and pathologic findings support this hypothesis.

The age-adjusted incidence rates of NSHL and MCHL are distinctive. NSHL preferentially affects adolescents and young adults. Males and females are affected almost equally. Mediastinal lymph nodes and low clinical stage disease are the rule. The following social factors correlate with an increased risk of NSHL: small family size, early birth order, a single-family home, fewer neighborhood playmates, higher maternal education, and higher paternal social class [455, 456]. These epidemiologic findings suggest that NSHL is the result of relatively late exposure to an infectious agent, analogous to the paralytic polio model. By contrast, MCHL is relatively more common in young children and older adults. Males are affected more often than females. The mediastinum is involved less often and advanced stage of disease is more frequent. Social factors that correlate with increased risk of MCHL are the converse of NSHL: large family size, late birth order, more neighborhood playmates, lower maternal education, and lower paternal social class [455, 456]. These findings suggest that early exposure to an infectious agent may play a role in causing MCHL.

EBV, commonly identified in MCHL, may help to explain some of these epidemiologic features. Children <5 years of age rarely develop HL. Perhaps in this age group children are exposed to EBV, particularly in poor socioeconomic conditions, but a latency period is needed prior to the development of HL as a result of primary infection. Cases of MCHL in adults and the elderly population may be the result of reactivation of EBV, perhaps secondary to immunodeficiency that occurs as a result of aging or other causes. The relatively low prevalence of EBV in NSHL suggests that EBV is not necessary for NSHL to develop and that another infectious agent or other factors may be responsible. The marked heterogeneity of the pathologic and clinical findings within NSHL also raises the possibility that this disease may result from more than one infectious agent.

It also now seems clear that the two-disease hypothesis was too simple. Nodular lymphocyte predominant disease is a third disease included under the rubric of HL. The independent status of NLPHL as a third disease is supported by its unique immunophenotype, molecular features, and distinctive clinical behavior, characterized by late relapses. It seems likely that LDHL is an end stage of other types of HL. The place of LRCHL is not clear, as it has features intermediate between classical HL and NLPHL.

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41 Immunology of the Lymphomas

Christopher Sequeira and Howard Ozer

Introduction

In the past 50 years, important discoveries have helped our current understanding of the immune system and its neoplasms. Early investigations led to the identification of distinct subpopulations of lymphocytes, the T (thymic-derived), B (bursa-equivalent), and natural killer (NK) lymphocytes which were not necessarily discernible by morphology and had differing functions [1, 2]. Cell-mediated immunity was found to be a function of T lymphocytes, while antibody responses (humoral immunity) were found to be a function of B lymphocytes. T and B cells were differentiated by the presence of certain antigens or proteins on their cell surfaces, specifically erythrocyte-rosette receptors (T) and surface immunoglobulins (B). It was later noted that different stages of lymphocyte maturation could be distinguished by the combination of particular antigens, generally glycoproteins, present on the cell surface, termed differentiation antigens. These antigens may be identified by specific, pure hybridoma antibodies (monoclonal antibodies) raised against them. The discovery of surface antigens on lymphocytes revolutionized the study of lymphoma. The combination of surface antigens (now termed cluster designation or CD group) present on a particular cell was designated its immunophenotype. The use of immunophenotypes has been helpful in classifying lymphomas into groups of B- or T-cell types and has provided insight into lymphocyte maturation. The result has been the development of new schemas of lymphocyte differentiation and new histologic classification systems attempting to correlate histology with phenotype. The progress in technology at the molecular level now permits detection of the earliest commitment to B- and T-lymphoid differentiation and serves to confirm immunophenotypic data.

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As more has become known about lymphocyte differentiation and function, the role of abnormal immune responses in the development and perpetuation of lymphomas has been exploited for therapeutic potential. The efficacy of therapy with biologic agents that are employed to improve or restore abnormal immune function continues to be an area of intense ongoing investigation.

Immunologic Phenotyping

The phenotypic analysis of lymphomas has yielded practical applications for diagnosis and furthered our knowledge of lymphocyte differentiation. Understanding the development of lymphocytes from stem cells to mature, immunocompetent T and B cells is made easier by the demonstration of lineage-specific differentiation antigens. However, the critical initial step is to subclassify lymphoproliferative neoplasms within the T- and B-cell category because several of these differentiation antigens, for example, immune-associated antigen (Ia) determinants, are not necessarily T- or B-specific. The ability to detect the commitment to B- or T-cell lineage at an earlier stage is now possible through analysis of immunoglobulin gene or T-cell receptor (TCR) gene rearrangements.

In 1832, Thomas Hodgkin provided what is thought to be the first description of a patient with lymphoma [6]. More descriptions into the twentieth century of patients with enlarged lymph nodes and pathologic lymphoid follicle proliferation eventually led to a classification of approximately 600 patients by Gall and Mallory in the early 1940s [7]. They described follicular lymphoma (FL) as a distinct entity based on morphologic and clinical characteristics and described its ability to progress by histology in a series of 63 cases reported in 1941 [8]. The advent of the Rappaport classification in 1956 brought a novel approach to the description of lymphomas categorizing them as either “nodular” or “diffuse,” the latter noted to have a worse prognosis [9, 10].

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Emerging techniques in immunophenotyping were applied to these entities within the Rappaport classification identifying their cell of origin as a follicular B cell [11]. The propensity of sheep erythrocytes to form rosettes around human T lymphocytes was utilized to identify Sezary cells and a proportion of lymphoblastic lymphomas to be of T-cell origin [12, 13]. This "E-rosette receptor" was eventually identified as CD2, a differentiation antigen found on nearly all T cells [14, 15]. The criteria utilized to establish the B-cell nature of a particular lymphoma are highly accurate but complicated. Any cell capable of synthesizing immunoglobulin, even by genetic criteria, is considered of B-cell origin. This can be rigorously demonstrated by removing all existing surface immunoglobulin. In lieu of this time-consuming procedure, with its limitations of cell viability, the demonstration of surface or cytoplasmic immunoglobulin showing restriction of the light chain (only κ or λ) is considered sufficient to conclude that a malignant cell is of B origin [16]. The demonstration of surface immunoglobulin alone is not sufficient to identify B cells conclusively because adsorption or uptake by Fc receptors of exogenous immunoglobulin in the serum will give a false-positive result, although in these cases there will be no light chain restriction [17, 18]. Conversely, adsorption of exogenous cytophilic immunoglobulin may give a falsely polyclonal finding for malignant monoclonal B cells that also express Fc receptors. In both these instances, the demonstration of J chains, present only in cells that synthesize immunoglobulin, may clarify the nature of the cell in question [19, 20]. The development of monoclonal antibodies through the use of hybridomas in 1975 [21] helped revolutionize the approach to lymphocyte and lymphoma immunophenotyping. The first monoclonal antibody targeting a human lymphocyte differentiation antigen was developed in 1979 [22]. The antigen was expressed on normal thymocytes and was later identified as CD1a in the human leukocyte differentiation antigen system [23]. In an effort to organize the evolving discoveries of cell membrane antigens, the CD or "clusters of differentiation" nomenclature was developed. To date, 350 CD designations have been determined and now include not only those found on the cell membrane, but also those that reside in the nucleus or cytoplasm [24].

Aside from distinguishing between T- and B-cell lymphomas, phenotyping also appears to shed light on the differentiation of normal lymphocytes and the malignant transformation of lymphomas. Phenotyping typically shows persistence of the major phenotype (T- or B-cell type) with few changes in surface receptors and identical heavy and light chain type in histologically transformed specimens [25–29]. The occasional patient in whom the heavy chain changes on subsequent biopsies may be a result of natural maturation, processes similar to those observed in B-cell cultures where μ/γ progresses to γ/α alone or switches to a γ/α heavy chain [30]. The prevailing theory is that the development of lymphoma cells parallels that of normal lymphoid tissue, with blocks occurring at various stages of development. This results in the heterogeneity of immunologic phenotypes in non-Hodgkin's lymphoma [27]. Thus, comparison of phenotypes from normal thymus, tonsils, and peripheral blood with lymphomatous tissue should be similar at equivalent stages of development. This theory fails to account for cells for which no normal counterpart has clearly been identified, as with hairy cell leukemia. The Lukes–Collins schema of B-cell maturation would place small, well-differentiated lymphocytes as early B cells with progressive development through follicular center cells (small cleaved to large cleaved to small noncleaved to large noncleaved) to immunoblastic lymphoma or multiple myeloma [31]. The T cells would mature from pre-T to convoluted thymic cells to mature T cells to functionally active effector cells (i.e., Sezary cells). The transformation from CLL to Richter's could thus be considered progression to a more mature cell type via a "switched on" transformation process, while conversion of MM to an immunoblastic cell type would result from dedifferentiation [32, 33]. The B schema of development differs little from previous convention except in the classification of CLL as an immature cell as opposed to a very mature cell. In this ordering, the intermediately mature forms are more virulent than the least and most differentiated forms. Habeshaw, using the Kiel classification, proposed a very similar sequence to that of Lukes. The B-cell schema he proposes begins with the pre-B-cell ALL cells progressing to CLL lymphocytes, then to germinal center cells (centroblastic/centrocytic), and finally immunoblastic cells or plasma cells. T-cell differentiation progresses from prethymic to thymic to mature T and finally to functional peripheral T cells. The maturation and proliferation schema constructed by Minowada et al. [27] from 55 long-term cell lines concurred with that of Lukes and Habeshaw.

The phenotypic maturation begins with the progenitor B cell, which possesses terminal deoxynucleotidyl transferase (TdT) activity, has Ia positivity, and may have immunoglobulin heavy chain gene rearrangement [34]. As the B cell matures to the pre-B stage, it retains TdT activity and sequentially acquires the CD10, CD19, and CD20 antigens and genotypically rearranges the immunoglobulin heavy chain gene and then the light chain gene. The early B-cell stage is characterized by loss of TdT activity and the CD10 antigen and acquisition of the mouse-rosette receptor and small amounts of surface immunoglobulin. Intermediate B cells express receptors for complement and Fc. These latter two stages correspond to the arrested developmental stages of CLL and small lymphocytic lymphomas. Mature B cells show variable expression of CD10 and CD21 and loss of the mouse erythrocyte receptor. These antigens (CD10, CD21) are lost in the plasmacytoid B-cell stage, at which point PC-1

and PCA-1 begin to appear. The terminal stage of B-cell maturation, the plasma cell, expresses PC-1 and PCA-1 and may express Ia but loses complement and Fc receptors and the pan-B antigens CD19 and CD20.

T-cell maturation progresses through three stages of thymocyte differentiation: (1) the early thymocyte (group I), which expresses CD5 and CD7 with or without CD2; (2) the common thymocyte (group II), which is CD2, CD5, and CD7-positive and now expresses CD1, CD4, and CD8 with variable CD3 positivity; and (3) the mature thymocyte (group III), which no longer expresses CD1 nor coexpresses CD4 and CD8. The mature thymocyte expresses the pan-T antigens CD2, CD5, and CD7 and the CD3 antigen. Mature T cells also express pan-T antigens CD2, CD5, and CD7 and CD3 with variable Fc receptor positivity. As with mature thymocytes, they should express either CD4 or CD8 [34].

The use of a panel of monoclonal antibodies to identify immunophenotypic patterns, not simply light chain restriction or E-rosette formation, has redefined the approach to lymphoma designation. Flow cytometry, which allows the immunophenotyping of individual cells in suspension for the presence or absence of antigens of interest, has helped us to improve diagnosis, classification, prognostication, and therapeutic decision-making. Antigens to be evaluated from a particular specimen depend on the type of specimen, the clinical history, as well as results of prior flow cytometric testing. Specific recommendations regarding flow cytometry indications and applications were formulated in 2006 after international experts convened in Bethesda, Maryland [35].

Non-Hodgkin's Lymphoma, B-Cell Types

Immunoglobulin light chain restriction and aberrant antigen expression distinguish neoplastic mature B-lymphoid cells from normal cells. Ault [36] first described the use of cytofluorographic techniques to rapidly screen large numbers of cells for κ and λ immunofluorescent staining. The sensitivity of the original technique permitted detection of a monoclonal B-cell population constituting less than 10% of the cells screened. A comparison of healthy normal volunteers, hospitalized patients without neoplasms, patients with non-Hodgkin's lymphomas in complete remission, and those with active non-Hodgkin's lymphomas without and with a leukemic phase yielded the following data. The hospitalized patients had relative increases of both κ- and λ-positive cells, but the κ/λ ratios were not significantly different in either light chain type to indicate the presence of a monoclonal population. Four of eight patients in complete remission and 44% of patients with active disease but without a leukemic phase had abnormal ratios of κ to λ but in only 32% of active nonleukemic non-Hodgkin's lymphoma patients was a distinct monoclonal pattern seen. All of the patients with leukemic non-Hodgkin's lymphoma had monoclonal patterns, that is, a distinct clonal excess. Other series utilizing more sensitive techniques subsequently confirmed clonal excess in up to 100% of B-cell non-Hodgkin's lymphoma patients with leukemia, CLL, multiple myeloma (MM), and Waldenström's macroglobulinemia (WM). Approximately 30% of B-cell non-Hodgkin's lymphoma without identifiable circulating leukemic cells revealed clonal excess [26, 37, 38]. The same techniques have also demonstrated clonal excess in pleural effusions with and without positive malignant cytology and in bone marrow biopsy specimens with no evidence or equivocal evidence of malignant involvement. In all instances, there was concordance among peripheral blood, bone marrow, lymph node, and pleural fluid as to light chain type [37, 39]. Clonal excess occurs more frequently in diffuse than follicular lymphomas, and more often in advanced stages of disease and in active or progressing disease [40].

While light chain restriction has typically been utilized as a marker of clonality, its presence has been detected in polyclonal B-cell populations [41]. Lambda immunoglobulin light chain restriction has been detected in tonsillar specimens of children as well as in patients with multicentric Castleman's disease [42, 43]. Similarly, monoclonal light chain class-restricted populations have been detected in follicular hyperplasia, such as that seen in patients with HIV, without neoplasia [44]. The presence of immunoglobulin light chain restriction is not universally indicative of monoclonality, and monoclonality of some lymphoid populations may not be synonymous with a neoplastic process. The importance of interpreting immunophenotyping in the context of other clinical and morphological findings cannot be overemphasized.

When the population of light chain-restricted B lymphocytes is large, abnormalities in the kappa–lambda ratio are found. However, small populations of clonal cells that are part of a polyclonal B-cell population may not be reflected in the kappa–lambda ratio. Evaluating those cells with a particular phenotype may more accurately detect a monoclonal population. Further complicating staining interpretation is the potential for nonspecific or cytophilic binding of antibodies. This is particularly evident in antibody binding to damaged or apoptotic cells. Cytophilic binding can be circumvented through the selective evaluation of only those cells expressing B-cell-associated antigens. Incubation of cells with a blocking reagent prior to anti-light chain antibody staining can minimize nonspecific staining.

Another potential pitfall of flow cytometric analysis is the possibility of not detecting an abnormal B-cell population in a tumor sample. False-negative results may occur secondary to sampling error, particularly in the case of tissue specimens in which the area of interest must be evaluated to appropriately identify areas for testing. Loss of cells during processing can vary depending on technique and cell type. Evaluating

more than one B-cell antigen and performing a cursory evaluation of all cells in the sample can avoid missed detection of abnormal B cells. Prior therapeutic treatment with the anti-CD20 antibody rituximab may yield neoplastic B cells devoid of CD20 [45]. B cells rarely may not demonstrate any surface immunoglobulin and complete absence by flow cytometry has been associated with a diagnosis of B-cell lymphoma in one series [46].

The use of flow cytometry has changed the approach to diagnosis in the modern era and, while not used in the World Health Organization (WHO) classification, does allow us to categorize lymphomas based on their immunophenotypic characteristics [47]. The determination of CD5 and CD10 expression can assist diagnostic efforts in combination with morphologic characteristics.

CD5−/CD10− Lymphomas

Those B-cell neoplasms positive for CD5 and negative for CD10 primarily encompass chronic lymphocytic leukemia/small lymphocytic lymphoma (CLL/SLL) and mantle cell lymphoma [48]. Marginal zone lymphoma (MZL) and diffuse large B-cell lymphoma (DLBCL) are associated with this phenotype, though in only a minority. Less commonly, the phenotype has been noted in patients with lymphoplasmacytic lymphoma. CLL/SLL has a characteristic phenotype with weak CD20, CD22, CD79a, and surface immunoglobulin expression. It typically demonstrates a strong CD23 intensity and is usually negative for FMC-7 [49]. FMC-7 is a 105-kDa protein expressed on most normal B cells and has been shown to be of significant diagnostic value in distinguishing CLL from other B-cell neoplasms [50]. Despite this characteristic phenotype, other entities such as prolymphocytic leukemia and DLBCL must be excluded. Further immunophenotypic information in the setting of CLL/SLL including CD38 and ZAP-70 expression may be helpful for prognostication [51]. CD38 expression has been thought to be a marker of poor prognosis in patients with CLL. While initially touted as a surrogate marker of immunoglobulin heavy-chain variable region (IgV(H)) mutation status, the two appear to be independent prognostic indicators [52]. In one series, the median survival of CLL patients with unmutated IgV(H) genes and expressed CD38 was 8 years, compared with 26 years in those patients with cells that had mutated IgV(H) genes and negative CD38 expression. The median survival in samples with discordant results was 15 years [52]. Zeta-chain-associated protein (ZAP-70) is a 70-kDa tyrosine kinase that plays a role in T-cell receptor signaling [53]. Gene expression profiling of CLL cells determined that those cells with unmutated IgV(H) genes had differential expression of select proteins, one of which included ZAP-70 [53]. Measurement of this protein by flow cytometry has been utilized as a surrogate marker of IgV(H) mutational status [53–58].

Mantle cell lymphoma (MCL), which also demonstrates a CD5+/CD10− phenotype, typically expresses CD20 and surface immunoglobulin with moderate to bright intensity. In contrast to CLL/SLL, CD23 expression is usually absent or weak and FMC-7 is typically detected. However, its phenotype can be variable, so additional studies are usually required for appropriate identification. Cyclin D1, a key regulatory protein of the cell cycle, appears to play an important role in the pathogenesis of MCL as evidenced by the presence of translocations leading to its overexpression [58]. Reliable detection of cyclin D1 expression by flow cytometry to distinguish MCL from other B-cell neoplasms has not been possible despite a variety of technical approaches [59].

A minority of samples from patients with DLBCL will demonstrate CD5 expression. The morphology of the cells allows distinction from small CD5+ lymphoid malignancies, though exclusion of the blastoid variant of MCL should be undertaken. DLBCL expressing CD5 may signify transformation from CLL/SLL [60] or may have developed de novo [61]. The latter would include the extremely rare entity of intravascular large-cell lymphoma. In a series of 120 patients with de novo CD5+ DLBCL, a higher rate of CNS recurrence was noted with a more aggressive clinical course [62]. The association of CD5 expression with a worse prognosis may be due to its proposed ability to promote IL-10 production and its effect on B-cell receptor signaling with resultant apoptosis avoidance [63].

Marginal zone lymphoma expresses CD5 in approximately 5% of cases. It may present a diagnostic challenge when trying to distinguish it from CLL/SLL and other CD5-positive lymphoid tumors [64]. Lack of CD23 expression in most cases of marginal zone lymphoma may also complicate diagnostic efforts. The presence of CD138 and CD38 expression suggests plasmacytic differentiation, although this finding is not specific to marginal zone lymphoma and serves more to distinguish it from CLL/SLL [48]. Genotypic studies may help establish the diagnosis, though a unique genotype does not appear to exist in marginal zone lymphoma [65]. Extranodal marginal zone lymphoma involving mucosa-associated lymphoid tissue, known as MALT lymphoma, can arise in a number of sites including the stomach, the salivary glands, or the thyroid gland [66–68]. Over two-thirds of MALT lymphomas arise in the stomach; thus it is the site most commonly studied. That gastric MALT lymphomas reproduce features of small intestinal Peyer's patches eventually led to the discovery that its development, at least initially, is antigen-dependent [69, 70]. Histologic features suggesting a role of the immune system in this disease include the invasion of lymphoma cells into germinal centers of normal B-cell follicles and the presence of plasma cell differentiation [71]. The association between gastric MALT

lymphoma and *H. pylori* infection was first proposed by Isaacson and colleagues when they demonstrated the bacteria in the majority of samples they studied [72]. Their work was later confirmed by others [69, 73, 74]. An immunological response playing a role in the development of MALT lymphoma was initially suggested again by the work of Isaacson and others [75]. Their key finding that the death of lymphoma-associated cells (T and B cells, macrophages) in culture could be abrogated by exposure of these cells to *H. pylori* preparations provided preliminary evidence of a causal relationship beyond the mere presence of the bacteria in tumor samples. Hussell and colleagues made the important observation that *H. pylori* was able to stimulate proliferation of tumor-infiltrating T cells and not lymphoma cells such that development of lymphoma appeared to be dependent on the recognition of *H. pylori* by T cells resulting in B-cell activation [76]. This supported the observations that MALT lymphomas of the stomach are usually not disseminated and that treatment of the infection results in tumor regression in about 75% of patients with early stage disease [77, 78]. The infiltration by CD4+ T cells expressing CD28 and CD69 and demonstrating interleukin-4 production and not interferon-γ suggests a Th-2-driven B-cell proliferation [79, 80]. Antigenic stimulation of the B-cell receptor may lead to NF-B activation with subsequent cell proliferation as supported by the observation that MALT lymphoma cells demonstrate somatically mutated surface immunoglobulin [81, 82]. Sequencing of rearranged immunoglobulin heavy chain genes in MALT lymphoma samples by Du and colleagues demonstrated intraclonal variation suggestive of ongoing mutation [83]. The identification of a target antigen has been elusive, although recent results demonstrate that recombinantly expressed MALT lymphoma antibodies are polyreactive and bind with some affinity to both self- and nonself antigens [84].

CD5−/CD10+

The most commonly encountered neoplasms expressing CD10 without CD5 expression include DLBCL and follicular lymphoma. Burkitt's lymphoma is also included in this immunophenotypic entity.

Diffuse Large B-Cell Lymphoma

Classification of DLBCL by gene expression profiling has identified two subgroups: germinal center (GC-DLBCL) and activated B-cell (ABC-DLBCL) diffuse large B-cell lymphoma [85]. A heterogeneous third subtype, termed type 3, was identified with genes in common with neither GC- or ABC-DLBCL [86]. In a series of 274 patients, germinal center DLBCL cases were associated with a greater rate of 5-year survival than both ABC-DLBCL and type 3 subgroups [86]. GC-DLBCLs appear to arise from B lymphocytes derived from germinal centers, while ABC-DLBCLs are thought to develop from postgerminal center-activated B cells [85–87]. Another distinct molecular subtype of DLBCL, primary mediastinal B-cell lymphoma (PMBL), has been noted to have significant similarity to Hodgkin's lymphoma (HL). The higher expression of over one-third of characteristic genes that distinguished PMBL from other DLBCLs was detected in HL cell lines [86]. Clinical parallels found in PMBL and HL including prevalence in young women with a predilection for mediastinal or thoracic involvement as well as their genotypic similarities suggesting a possible relation are yet to be molecularly defined [86].

In GC-DLBCLs, translocations involving the transcription factor BCL-6 are characteristic, and were originally identified due to their association with DLBCL [88]. BCL-6 is a regulator of GC B-cell development and is expressed by GC B cells, centroblasts, and centrocytes [89]. Aberrant BCL-6 expression appears to promote centroblast differentiation resulting in a proliferative phenotype [89].

ABC-DLBCL has been associated with aberrant NF-κB signaling as well as with constitutive activation of STAT3 [90, 91]. BCL-6 is thought to repress STAT3 expression in normally functioning B lymphocytes [90]. ABC-DLBCLs demonstrate decreased BCL-6 expression with resultant increase in STAT3 levels. Using siRNA, Ding et al. demonstrated that inactivation of STAT3 inhibited lymphoma cell proliferation and promoted apoptosis [90]. Increased NF-κB activity in ABC-DLBCL cells results in increased IL-6 and IL-10 secretion that can lead to constitutive STAT3 activation [90, 91]. BCL-6 is thought to repress STAT3 expression in normally functioning B lymphocytes [90]. Increased NF-κB activity in ABC-DLBCL cells results in increased IL-6 and IL-10 secretion which can lead to constitutive STAT3 activation [91].

ETS proteins are transcription factors that control the expression of genes involved in mechanisms such as cellular proliferation, differentiation, and apoptosis [92]. In the study of immunoglobulin class switch recombination (CSR) as a potential mechanism of chromosomal translocation in DLBCL, it has been suggested that ABC-DLBCLs have changes in the regulation of CSR leading to chromosomal translocations involving *BCL-6*, *MYC*, as well as *SPIB* (SPI-B transcription factor) [93].

SPIB belongs to the ETS family of transcription factors and is found in mature B cells, T-cell progenitors, and plasmacytoid dendritic cells [94, 95]. *SPIB* is integral to B-cell receptor signaling and appears to play a central role in germinal center reaction as evidenced by the smaller size, shorter duration, and greater proportion of apoptotic cells of germinal centers in *SPIB*-deficient mice [96]. The importance

of *SPIB* as a regulator of mature B-cell function is supported by its Blimp-1-mediated repression during plasmacytic differentiation [97]. That *SPIB* demonstrates much higher expression in ABC DLBCLs than in GCB DLBCLs and its translocation in the ABC DLBCL cell line OCI-Ly3 support a potential role in this DLBCL subtype [93].

Follicular Lymphoma

The clinical course of follicular lymphoma (FL) is typically indolent with median survival on the order of 8–10 years though there appears to be variability [98, 99]. BCL-2 overexpression noted in the majority of FL samples leads to impaired intrinsic and mitochondrial apoptotic pathways [100]. Dysregulation of BCL-2 arising from the characteristic (t14;18)(q32;q21) translocation does not appear to be in and of itself sufficient for transformation to malignancy. In a *BCL-2/Ig* transgenic mouse model, the development of lymphoma occurred in only a small portion of animals [101]. Follicular hyperplasia preceded the transformation to large-cell lymphoma with progression from a polyclonal population to one that was monoclonal before the change in histology to a large-cell phenotype. This progression with a long latency suggested the necessity of additional changes for lymphoma development, not just the presence of BCL-2 overexpression. In fact, the translocation promoting BCL-2 production has been noted in 55% of peripheral B lymphocytes from patients without lymphoma and appears to increase in incidence with age [102]. Over half of lymph nodes and tonsillar specimens with follicular hyperplasia demonstrated bcl-2/JH rearrangements in patients with no evidence of FL [103]. The lack of lymphoma development in BCL-2 transgenic mice with the observation of the characteristic translocation in normal populations supports the notion that additional changes are necessary in the pathophysiology of FL.

In vitro, FL cells have a short survival on the order of 24 h. Exposure of FL cells to cytokines can prolong cell survival to 1 week and beyond [104]. The microenvironment appears to play a crucial role in FL genesis, particularly in light of prior work demonstrating the inability of genetic changes alone to bring about disease. The germinal center where antigen-activated B cells differentiate to produce plasma cells and memory B cells may be a source of FL genesis. Follicular dendritic cells (FDC), traditionally known to function as antigen-presenting cells, also appear to inhibit germinal center B-cell death and promote cellular interaction and proliferation [105]. FDCs demonstrate expression of the complement receptors CD21 and CD35 and the immunoglobulin receptor CD23. Production of the chemokine CXCL3 by FDCs is high [106]. Expression of these markers by mature FDCs supports a surrounding network of T cells. Loss of CD21 receptor in FDCs appears to be related to large-cell lymphoma transformation associated with a poor prognosis [107]. Poor prognosis FL characterized as resistant to anti-CD20 treatment and early transformation to DLBCL possess genotypes demonstrating activation of mediators of cellular response including macrophages, dendritic cells, and activated T cells [108–110]. Unlike other B-cell lymphomas, FL does not appear to derive from antigenic stimulation. Rather, the overexpression of BCL-2 confers a growth advantage. Additional cytogenetic mutations (for example −6q and +1q) result in eventual activation of FDC and T cells and the development of an aggressive phenotype [111]. In this model, B-cell localization may be defective with tumor cell deposition within follicles. Clonal evolution may proceed at an increased rate resulting in early transformation. The microenvironment in better prognosis FL patients is more consistent with an inactive follicle and maintains CD21/CD35 and CD23 expression with greater reliance on anti-apoptotic signals for its malignant phenotype. In contrast to its more aggressively behaving counterpart, the genome in good prognosis patients appears to remain more stable until transformation [112]. This effect of the tumor microenvironment on FL pathogenesis has been supported by gene expression profiling of immune bystander cells. In a series of nearly 200 FL samples at diagnosis, two subsets of patients with differing gene expression signatures were discovered possessing distinct clinical behaviors [109]. As the differing genotypes were derived from bystander cells, they were termed immune response (IR) 1 and 2. IR1 consisted of increased expression of T-cell markers including *CD7, CD8B1, ITK, LEF1*, and *STAT4* and macrophage-derived genes including *CTN1* and *TNFSF13B*. The IR1 gene signature was associated with an improved overall survival. The IR2 signature consisted of macrophage and FDC-associated genes including *TLR5, FCGR1A, SEPT10, LGMN*, and *C3AR1*. Those patients whose tumors displayed the IR2 signature demonstrated a worse clinical outcome. In another series of 99 FL advanced stage patients, those with more than 15 CD68+ macrophages per high power field had a median survival of 5.0 years compared with 16.3 years in those patients with less than 15 CD68+ cells/hpf [113]. These results were confirmed by PCR and immunohistochemical analysis by Byers and colleagues [114] demonstrating an association of high numbers of CD68+ macrophages with worse outcomes. Increased CD7+ T cells were equated with longer survival. The presence of CCR1, a chemokine receptor indicative of monocyte activation during inflammation, predicted for a worse prognosis as well as transformation to DLBCL [110, 114]. The results of these analyses supported the developing notion that the host immune response plays a part in FL pathogenesis.

Burkitt's Lymphoma

Burkitt's lymphoma (BL), though more consistently so in its childhood form, is also among the category of CD5−/CD10+ lymphoma immunophenotypes. It is considered a highly aggressive lymphoma subtype and almost always demonstrates a Ki-67 index of 100%, a marker of cell proliferation. The BL variants include endemic, sporadic, and those associated with immunodeficient states. Like DLBCL, BL is also associated with a germinal center phenotype demonstrating CD77, a marker of the highly proliferative centroblast [115]. Within normal germinal centers, antigen-stimulated B cells proliferate. Their immunoglobulin genes are subject to somatic gene rearrangement and class switch recombination [116]. These processes are initiated by deamination mediated by activation-induced cytidine deaminase (AID) [117, 118]. Aberrant somatic hypermutation [119] can result in deregulated B-cell proliferation through characteristic translocations such as that of the *c-myc* gene into the Ig locus of BL [120]. The targets of c-myc are several and include regulation of cell proliferation, cell cycle control, and cell metabolism [121, 122]. Transgenic c-myc murine models have demonstrated its importance in the development of lymphoma, although those that develop are not of the germinal center phenotype [123–125]. Scheller and colleagues set out to elucidate the constitutive GC phenotype of BL cells and somatic hypermutation [126]. They were able to demonstrate that *c-myc* overexpression induces the cells to behave like centroblasts, results in expression of the key GC factors Bcl-6, E2A, and AID and also promotes ongoing somatic hypermutation in the cells. These features of human BL cells may thus be indicative of the high activity of the oncogenic transcription factor c-Myc, rather than a marker of differentiation stage of the cell of origin.

Though the association between Epstein–Barr virus (EBV) and BL has long been established, the mechanism of the infectious agent's capability to promote lymphomagenesis is still being elucidated. The overexpression of c-myc appears to be independent of EBV infection, though a potential relationship has been proposed as a means of pathogenesis [127]. Those areas of the world with endemic BL demonstrate *P. falciparum* malaria as a cofactor for EBV-mediated lymphoma development [128]. The immunosuppressive effect of malarial infection [129–131] appears to be the reason why children in endemic areas have extremely high EBV loads in their blood that increase during acute malarial infection [131–133]. *P. falciparum* also appears to have a proliferative effect on germinal centers. C-myc translocations in these germinal centers resulting in aberrant immunoglobulin gene rearrangements would normally be censored, but may be rescued by the growth advantage conferred by EBV infection. While HIV-infected patients do not demonstrate as high EBV levels as in patients with a malarial infection, a similar mechanism of B-cell activation is hypothesized [134].

The immune mechanisms involved in BL development in the setting of EBV infection have been postulated for quite some time. Important observations that EBV-infected cells can develop varying latent states in which cells are infected but viral particles are not replicating has resulted in a better understanding of why EBV infections results in lymphoproliferative disease in only a proportion of patients [135–137]. Resistance to HLA class I restricted cytotoxic lymphocytes by EBV-infected BL cells has suggested an escape from immune surveillance [138–141]. BL cells express reduced levels of adhesion molecules such that recognition by CD8+ T cells is impaired [135, 140, 142]. Enhanced antigen presentation in some long-term carriers allows EBV persistence without the development of lymphoproliferative disease [127]. The ability of BL cells to evade apoptosis by the promotion of certain viral latent genes appears to be crucial to its B-cell growth-transforming capacity. Latent membrane protein 1 (*LMP1*), the first of these latent genes to be identified, promotes the production of Bcl-2, Mcl-1, and bfl-1 with resultant apoptosis circumvention [143–146]. The development of BL in the setting of EBV infection appears to rely on a variety of viral properties providing a growth advantage in transformed cells, the mechanisms of which are slowly being elucidated.

Non-Hodgkin's Lymphomas, T-Cell/NK-Cell Types

The T-cell non-Hodgkin's lymphomas comprise several distinct clinical and pathologic entities that are now also found, in most cases, to be immunologically distinct as well. The differentiation stages of T-cell lymphomas parallel leukemic forms closely and incorporate neoplasms from all stages of T-cell development, thymic to immunocompetent T-cell types. Despite the capacity to distinguish T-cell neoplasms phenotypically and functionally, the classification of T-cell neoplasms is difficult to establish and frequently requires genotypic (T-cell receptor or TCR rearrangement) confirmation. The WHO 2008 classification of lymphomas has retained the classification of peripheral T-cell lymphoma, not otherwise specified (PTCL-NOS) to describe some T-cell lymphomas for which distinctive genetic or biologic features have not yet been discerned [147–149].

Peripheral T-Cell Lymphomas

The normal cell of origin giving rise to peripheral T-cell lymphomas (PTCL) has not been definitively described [149]. PTCLs are thought to be derived from T cells expressing the

αβ form of the TCR [150]. Cells from PTCL-NOS samples demonstrate CD4 expression more commonly than CD8, though CD4+CD8+ and less commonly CD4–CD8− phenotypes can be found [151–153]. PTCLs comprise just over 10% of all lymphomas and generally include the three subtypes of angioimmunoblastic T-cell lymphomas (AITL), anaplastic large-cell lymphoma (ALCL), and peripheral T-cell lymphoma, not otherwise specified (PTCL-NOS). While the molecular profiling of B-cell lymphomas has enabled us to develop a better understanding of their heterogeneity and etiology, the genetic signatures of T-cell lymphomas are being slowly being elucidated.

Angioimmunoblastic Lymphoma

Also known as angioimmunoblastic lymphoma with dysproteinemia (AILD), AITL is characterized by cells with abundant cytoplasm, significant blood vessel formation, proliferation of follicular dendritic cells, and EBV-infected B lymphoblasts [154]. AITLs appear to have arisen from mature CD4+CD8− helper T cells [155]. Expression of the chemokine receptors CXCR3 and OX40/CD134 supports a Th1 cell of origin [156, 157]. Additionally there may be expression of a variety of markers including the transcription factor BCL-6 and the chemokines CXCL13 and CXCR5 derived from a subset of T-helper cells found in germinal centers known as follicular helper T cells (T_{FH}) [158–162]. Comparison of gene expression profiles of AITL and other PTCLs has demonstrated a significant amount of similarity suggesting a common pathogenesis [163]. In an analysis by Piccaluga and colleagues, genes characteristic for varying subpopulations of T cells were evaluated. AITL demonstrated a molecular signature most characteristic of T_{FH}, a finding confirmed by others [150, 163]. In concordance with other groups, Piccaluga demonstrated the relationship of PTCL-NOS and ALCL to T-central memory cells and T-effector cells, respectively [163, 164]. Overexpression of VEGF and VEGFR-2 in AILT samples suggests that derangement of angiogenesis is part of its pathophysiology and may explain the [163, 165]anecdotal responses reported with bevacizumab therapy. In an effort to develop a molecular classification approach to PTCLs to aid in diagnosis, one series analyzed over 140 tumor samples [166]. The classifier that was derived for AITL included genes associated with germinal B centers (including CD10 and Bcl-6), B-cell receptor-associated signaling and members of the immunoglobulin family as well as follicular dendritic cell (FDC) markers, cytokines, and their receptors. These findings demonstrated consistent involvement of FDCs and B cells in the AITL microenvironment with support of malignant T-cell growth as well as angiogenesis and immunosuppression stemming from cytokine signaling. When compared to the gene expression profiling results of de Leval et al. [150], Piccaluga identified significant overlap between the molecular signature of a series of PTCL cases and AITL enabling reclassification of 18% of PTCL-NOS as AITL [167]. While the proposed cell of origin in AITL is the T_{FH} cell, differences from normal T_{FH} cells including high CD10 expression and low CD57 expression have been noted [168]. SLAM-associated protein (SAP) is expressed by the gene *SH2D1A* (*SAP/DSHP*) [169]. Expressed in CD4 and CD8 T cells as well as NK cells and a proportion of B cells, SAP appears to play an essential role in Ag-specific CD4 T cells to develop appropriate follicular helper CD4 T-cell functions [170]. Despite the elevated expression of SAP and SLAM in AITL samples, follicles are underdeveloped and not populated with neoplastic T cells, suggesting that the lymphoma cells may be at a prefollicular stage of development [166]. Secretion of IL-6 and IL-21 by malignant T_{FH} cells may promote B-cell differentiation resulting in the plasma cell infiltrate seen in AITL [166, 171] with resultant hypergammaglobulinemia and autoimmune features characteristic of the disease.

Anaplastic Large-Cell Lymphoma

Anaplastic large-cell lymphomas (ALCL) make up about 12% of all T-cell lymphomas [172]. The heterogeneity of ALCL has resulted in much speculation regarding its etiology [173, 174]. The strong expression of CD30 in ALCLs and the distinction of the disease into subsets based on chromosomal translocations involving the anaplastic lymphoma kinase (ALK) gene significantly helped diagnosis [175–177]. Differing genetic aberrations in ALK+ and ALK− ALCLs resulted in their consideration as different entities in the WHO's most recent classification [47, 163, 178–180]. Currently, no immunophenotypic or molecular profiles exist to differentiate ALK-negative ALCL, leading to the proposal that it may be more accurately described as belonging to the PTCL-NOS category [181, 182]. However, work published by Piva and colleagues demonstrates that all ALCL may be genetically differentiated from PTCL-NOS, and they have reported a molecular signature distinct to ALCL ALK-positive neoplasms [183]. The discovery of differential expression of 30 genes in both ALK-positive and ALK-negative ALCLs of which only a few are associated with ALK signaling also suggests a possible common precursor for both subtypes of ALCL. Defective expression of T-cell receptors is found in all subtypes of ALCL also supporting the notion that ALK-positive and ALK-negative subsets, though with distinctive characteristics, may share similar etiology [184]. Furthermore, some ALK-negative ALCLs express proteins such as phosphor-STAT-3 typically associated with ALK-positive tumors [183]. The distinction of

ALK-negative ALCLs from PTCL-NOS may become more feasible with molecular signatures thus allowing improved therapeutic approaches. In the gene classifiers derived by Iqbal and colleagues, although a signature to distinguish ALK-positive from ALK-negative subsets was not determined, the latter was distinguishable from PTCL-NOS [166]. Expression of TCR signaling associated genes was lower in ALK-negative samples compared with PTCL-NOS, and the genes for the cytokines IL-20 and IL-9, involved in angiogenesis and Jak3 activation, respectively, demonstrated higher expression. In the ALK-positive subset of samples, the expected ALK mRNA was preferentially detected as well as IL-26 and IL-31RA, which can activate STAT3. STAT3 expression promotes the expression of IL-17A, IL-17F, and IL-22 via the retinoic acid receptor-related orphan receptor (RORγ). The expression of IL-17A and IL-17-F, both T_H17-associated molecules, may signal deranged activation of T_H17-cell differentiation by abnormal cytokine secretion [166].

Adult T-Cell Leukemia/Lymphoma

Adult T-cell leukemia/lymphoma (ATLL) has long been associated with the human T-lymphotropic virus type-1 (HTLV-1), a delta retrovirus that is estimated to infect 15–20 million people, globally [185, 186]. Approximately 2–5% of viral carriers will eventually develop ATLL [187]. ATLL manifests four to seven decades after infection supporting the notion that genetic or epigenetic events are necessary in the lymphoma's pathogenesis [188]. HTLV-1 expresses multiple gene products, one of which (TAX) appears to play a particularly important role in the promotion of infected cells and has been shown to complex with over 100 cellular proteins [189]. Multiple cellular pathways are stimulated by TAX including CREB/ATF, NF-κB, and AP1, all of which are intimately involved in T-cell proliferation and transformation [189]. In cellular models with defective TAX, lymphoma generation in vitro and in vivo is impaired [190]. Gene expression analysis of HTLV-1-infected and TAX-expressing cells has enabled identification of HTLV-1/TAX-regulated genes [191–194]. Included among these genes were those that regulate T-cell proliferation such as IL-2, IL-15, granulocyte-macrophage colony stimulating factor (GM-CSF), and tumor necrosis factor-α (TNF-α) [185, 186, 195].

A foxhead box protein 3 (Foxp3) represents a T-cell marker of Treg cells. It is a master gene regulating development and differentiation of CD4+/CD25+ Treg cells [196–199]. Adult T-cell leukemia cells, thought to represent post-thymic T cells, demonstrate CD4 and CD25 expression [200]. Foxp3 is present in adult T-cell leukemic cells as well as in HTLV-1-infected cells in asymptomatic carriers [201, 202]. Furthermore, infection of Treg cells by HTLV-1 has been shown to affect Foxp3 expression and thus suggests a potential role in the pathogenesis of adult T-cell leukemia [203–205]. Interestingly, in the development of a gene expression classifier to distinguish PTCLs, Iqbal and colleagues did not demonstrate an upregulation of Foxp3 mRNA, although overexpression of CD25 was seen [166]. In this series, when their classifier was applied to PTCL-NOS, three cases were reclassified as ATLL and were confirmed as arising from regions endemic for HTLV-1. Viral transcripts encoding the TAX protein were detected in these tumor samples and all three patients demonstrated a survival of less than 3 years, consistent with ATLL.

Cutaneous T-Cell Lymphoma

Of the heterogeneous cutaneous T-cell lymphomas (CTCLs), mycosis fungoides (MF) and Sezary syndrome (SS) are the most common entities. Clinically, MF is characterized by skin patches and plaques, whereas SS manifests as leukemic T cells and erythroderma [206, 207]. Although both MF and SS are clonally derived T-cell malignancies and demonstrate CD4+, CD45RO+, CLA+ (cutaneous lymphocyte antigen) expression, genomic differences exist suggesting a variable pathogenesis for the two conditions [208]. The etiology of CTCL has not yet been defined. Early stage disease is characterized by an inflammatory patch or plaque of the skin and demonstrates an infiltrate of reactive, nonmalignant T cells [209]. This T-cell infiltrate expresses CLA and CCR-4 [210, 211]. With progression of disease, the malignant T cells outnumber reactive cells in the dermis with increased atypia [211]. In a series of 62 CTCL samples, gene expression profiling revealed three "clusters" with divergent clinical behaviors [212]. Cluster 1 samples, associated with poorer prognosis, demonstrated lymphocyte clonal expansion with changes consistent with decreased T-cell receptor heterogeneity. Longer follow-up confirmed the accurate prognostic information derived from the three clusters [213]. Among the patients with stage I disease, those with cluster 1 or cluster 3 genes demonstrated a statistically significantly worse prognosis while those with a cluster 2 profile did not progress during the 6 years of follow-up. Analysis of select genes in this series revealed expression of genes associated with Th-17, a recently identified CD4+ T-helper cell with pro-inflammatory capabilities [214]. Others have shown malignant cells that share common features with T_{reg} cells as defined by expression of Foxp3 and CTLA-4 [215, 216]. Although these cells are thought to be dysfunctional [217], analysis of peripheral blood mononuclear cells (PBMC) from patients at varying stages of CTCL/MF has shed some light on potential pathogenic mechanisms [218]. Cytokine expression appears to diminish with advancing disease stage

suggesting defective immune response associated with disease progression. It remains unclear whether the cell of origin in CTCL is the T_{reg} cell or CD4+ T cells that adopt characteristics of T_{reg} cells [219].

While the cell of origin remains to be determined, also of interest is the mechanism by which malignant T cells localize to the skin to cause disease characteristic of CTCL. Chemokines, which are chemotactic, are thought to play an important role in the pathophysiology of CTCL [219]. Their receptors are cell-surface, G-protein-coupled receptors, of which there are reported to be 19. Varying expression of chemokine receptors on T-cell subsets results in differential responses to chemokine-stimulated T-cell migration such that expression of chemokines by epidermal keratinocytes can result in epidermal T-cell infiltration [220]. Chemokines, such as CCR-4 ligand produced by dendritic cells, may also play a role as malignant T-cell chemoattractants as suggested by the conjugation of dendritic cells with malignant T cells in Pautrier's microabscesses, characteristic of MF [221]. Expressed on about 25% of normal CD3+,CD4+ T cells, CCR-4 demonstrates much higher expression on malignant cells in MF and SS [219, 222, 223]. A ligand of CCR-4, CCL-17 is produced by keratinocytes, endothelial cells, and dendritic cells and is overexpressed in the dermis of patients with MF [224]. Similarly, CCL-27, a CCR-10 ligand, is less frequently expressed on normal circulating T cells but is increased in CTCL cells [222, 223]. The expression of CCL27 in normal keratinocytes suggests it may play a tropic role in bringing malignant cells to the dermis.

The dipeptidylpeptidase, CD26, is downregulated in MF and SS cells [225]. Inactivation of CXCL-12 by CD26 is thus impaired. CXCL-12 is a ligand of CXCR-4 and is produced by stromal cells and fibroblasts located in the dermis. In cell lines derived from patients with SS, absence of CD26 promoted chemotaxis that was mediated by unimpaired CXCL-12 [224]. Introduction of soluble CD26 inhibited migration via CXCR-4, suggesting a role of the CXCR-4/CXCL-12 axis in the epidermotropism of SS as regulated by CD26.

Natural Killer/T-Cell Lymphoma

Natural killer/T-cell lymphoma (NK/T) is a rare neoplasm of NK and NK/T cells. The WHO classification includes three subtypes of NK malignancies: extranodal NK/T-cell lymphoma, NK leukemia, and a blastic variant [226]. Extranodal NK/T-cell lymphoma (ENKL) is typically found in the nasal cavity or adjacent structures and is characterized by an aggressive disease course in advanced stages with chemotherapy resistance. It appears to be related to EBV infection as evidenced by geographic patterns of incidence.

It is thought that EBV infection is not merely associated with ENKL but plays a role in its pathogenesis. Normal nasopharyngeal cells in patients with ENKL demonstrate EBV infection; however, comparative genomic analysis reveals heterogeneity in the viral genome in contrast to the single strain detected in malignant cells from the nasopharynx [226]. Furthermore, the EBV genome within ENKL cells demonstrates deletion of *LMP-1* in nearly 100% of cases studied while normal cells carried the wild type. The level of circulating EBV DNA in patients with ENKL appears to be prognostic with high titers indicating a worse survival with treatment resistance.

The mechanisms of disease in ENKL remain largely unknown. Recent attempts to delineate genetic alterations as a means of understanding the biology of the disease have been undertaken, although the rarity of the disease and the predominance of necrosis in biopsy samples have been a hindrance. Huang and colleagues performed genomic analysis of nine samples of tissue from patients with ENKL and compared results with analysis of normal NK cells and cells from PTCL-NOS [227]. Comparison of ENKL cells with those from PTCL-NOS samples demonstrated in the former increased expression of genes related to NK-cell cytotoxic function. Granzymes (gzm), a family of serine proteases found in cytotoxic granules of NK cells, induce apoptosis in target cells [228]. The greatest increase in expression was noted in gzm H, which is constitutively expressed in NK cells and elicits apoptosis through the caspase-independent pathway [228, 229]. The predilection of ENKL for extranodal sites is corroborated by the findings of Huang in which downregulation of the chemokine receptor CCR7 and the upregulation of the chemokine ligands CCL27 and CXCL12 were noted, consistent with their roles in homing of cells to peripheral lymph nodes and to skin/intestine/bone marrow by CCR7 and CCL27/CXCL12, respectively. Other pathways implicated by the work of Huang and colleagues include deregulation of PDGFRα with resultant effects on AKT and Jak-STAT pathways, activation of NF-κB possibly through EBV infection, as well as increased expression of genes resulting in vascular invasion [227].

Hodgkin Lymphoma

The incidence of Hodgkin lymphoma (HL) in the United States is estimated to be 7400 cases per year [230]. Classical HL, comprised of nodular sclerosis, mixed cellularity, lymphocyte-depleted and lymphocyte-rich subtypes, represents 95% of HL cases with the remaining 5% made up of nodular lymphocyte predominant HL (NPHL) [47]. The tumor cells in classical HL (cHL) are termed Hodgkin and Reed–Sternberg (HRS) cells and in NPHL are known as lymphocyte predominant (LP) cells [47]. Aside from differing histologies, the cells of origin in cHL and NPHL are also distinct. In cHL, tumor cells are most likely derived from GC

B cells that have evaded apoptosis despite abnormal immunoglobulin V gene mutations in contrast to NPHL cells which result from antigen-selected GC B cells [231]. Although HRS and LP cells typically comprise less than 10% of cells in a tumor sample, efforts to analyze their molecular markers have been pursued in an effort to elucidate pathogenic mechanisms.

Somatic hypermutation of immunoglobulin V (IgV) heavy and light chains in HRS cells confirmed their origin from mature B cells that were of GC or post-GC differentiation [232]. Unlike most B-cell lymphomas that retain phenotypic characteristics of their cell of origin, HRS cells displayed characteristics of a multitude of hematopoietic cells [233]. Classic B-cell antigens such as the B-cell receptor (BCR), CD19, and CD20 are rarely expressed by HRS cells [234, 235]. Transcription factors that regulate certain B-cell-specific genes such as Oct-2, Pu.1, Bob1, and early B-cell factor (EBF) are also rarely seen [235–238]. Some transcription factors such as the helix–loop–helix transcription factors E12 and E47 are expressed, but concomitant expression of their inhibitors limits their function [236, 239, 240]. However, HRS cells express Pax5, a signal of commitment to B-cell lineage, although many of its target genes are not found [241].

Cases of HL in which T-cell markers are expressed have been reported and have been demonstrated as lacking Ig gene rearrangements and instead displayed T-cell receptor rearrangements suggesting T-cell origin [242–246]. In one series, gene expression analysis of cell lines from T-cell-derived HL, B-cell HL, and other B- and T-cell lymphomas (including CD30+ ALCL) demonstrated a gene expression profile in the T-cell HL similar to other HL cells supporting a common transformation pathway despite differing cells of origin [247].

Notch1, usually expressed in T cells, is also expressed in HRS cells and acts as a transcription factor favoring T-cell development over B-cell development in lymphoid precursors [248]. Its inhibitory effects on B-cell development center on its downregulation of the B-cell transcription factors E2A and EBF as well as promotion of ABF1 expression, a B-cell transcription factor inhibitor [248]. Jagged1, the activating ligand of Notch1, is found in cells from HL tumor samples supporting its role in HL biology [248].

The connection between LP cells in NLPHL and GC B cells was drawn with the identification in LP cells of classic GC B-cell markers including BCL-6 and cytidine deaminase. Furthermore, the association of LP cells in follicles with follicular dendritic cells and GC-type Th cells was also consistent with germinal center cell structure [232]. Gene expression profiling of LP cells in comparison with normal B cells and B-cell lymphomas suggested genetic similarity to late GC B cells that have adopted memory B-cell characteristics [249]. Though LP cells resemble follicular lymphoma cells in histology and in immunophenotype, gene expression profiling suggests a closer relationship with HRS cells than with FL cells [249]. Among the molecular similarities noted between HRS and LP cells is activation of the NF-κB pathway [249].

The role of EBV in the pathogenesis of a proportion of classical HL cases is well established. Approximately 40% of HRS cells have an EBV latent infection [232]. In EBV + HL cases, all HRS cells carry the virus. Expression of viral proteins including EBNA1, which is essential for viral replication, and LMP-1 and LMP-2a, which mimic CD40 and BCR, respectively, appears to be vital to lymphomagenesis in EBV-related cases [250, 251]. An important finding supporting the role of EBV in HL pathogenesis is the apparent requirement for EBV infection in those HL cases with loss of the BCR. It is postulated that EBV through LMP-2a expression can rescue from apoptosis B cells that have lost BCR. This rescue has been reproduced in vitro [251–253].

Constitutive activation of the transcription factor NF-κB appears to be required for survival of HL cells [254]. CD40, a member of the tumor necrosis factor family, is expressed by HRS cells and its ligand is expressed by T cells found in NHL tumors [255]. Activation of CD40 results in NF-κB activation. Other receptors expressed by HRS and potentially activating NF-κB include CD30, RANK, and Notch 1 [248, 256, 257]. Nearly half of classic HL tumors show amplification of the REL gene, which codes for a NF-κB factor [258, 259]. Mutations in the genes of NF-κB inhibitors IκBα (in about 15–20% of classic HL cases) and IκBε may also contribute to pathway activation. A recently identified tumor suppressor gene, *TNFAIP3*, is often mutated resulting in inactivation [260, 261]. The protein expressed by *TNFAIP3*, identified as A20, acts as an upstream inhibitor of the NF-κB pathway. Inactivating mutations have been identified in 30–40% of HL tumors studied [260, 261]. In vitro, restored expression of A20 in A20-silenced HL cell lines diminished expression of NF-κB pathway targets with a resultant decrease in cell survival [260, 261]. This phenomenon was also demonstrated in a murine model [260]. A similar analysis was undertaken to identify the role of NF-κB in LP cells but revealed only rare mutations in *NFKBIA* and *TNFAIP3* [232].

The microenvironment appears to play a crucial role in the pathogenesis of HL as evidenced by the difficulty in propagating HL cells in vitro and in vivo models. Secretion of chemokines and cytokines such as CCL5, CCL17, and CCL22 attract Th2 cells and IL-5, IL-9, CCL5, and CCL28 attract T_{reg} cells [232].

As described previously, the proportion of HRS cells in a given tumor is very small with the majority of cells represented by inflammatory lymphocytes, predominantly CD4+ T cells [262]. The pathogenic role of these infiltrating lymphocytes has been the subject of much investigation, and has been postulated to have a role in the suppression of effector

immune responses [263]. Suppression of immune response in HL has long been recognized and dates back to the recognition in 1902 by Dorothy Reed of diminished hypersensitivity response to intradermal purified protein derivative [264]. The propensity for graft rejection and the increased incidence of graft-versus-host disease after blood transfusion corroborated the immune suppression hypothesis [265–267].

The discovery of T_{reg} cells has sparked much interest in their potential role in many subtypes of lymphoma, including HL. T_{reg} cells are thought to mediate immune responses and control the body's response to foreign antigen such as in transplantation [268]. There are thought to exist two subtypes of T_{reg} cells, one which mediates its effects through cytokine release (IL-10 secretion) and the other through direct cell contact (CD4+CD25+) [269, 270]. Analysis of HL patient samples by Marshall and colleagues revealed the predominant cell populations in the HL infiltrating lymphocytes to be IL-10 secreting (or Tr1) and CD4+CD25+ T_{reg} cells (which were later found to express foxp3). The oft detected expression of CTLA-4 on HL lymphocytes also supports a T_{reg} infiltrate as it is a costimulatory molecule found on Tr1 and CD4+CD25+ T cells [271–273].

In a recent series published by Steidl and colleagues [274], gene expression profiling revealed the presence of CD68+ macrophages in the tumor infiltrate of classic HL samples to be associated with a worse survival. In fact, median survival in samples with the lowest quantity of CD68+ macrophages was not reached after over 16 years of follow-up compared with 2.7 years in those samples with the highest display of CD68+ macrophages. The exact role of macrophages in the pathogenesis of HL remains to be determined. This study also confirmed the favorable prognostic effect of CD20+ B cells in tumor samples, confirming the work of others [275].

Immunotherapy

The association of impaired immunity and neoplastic disease of the lymphoproliferative system is exemplified by clinical abnormalities, laboratory evidence of decreased immune responses in HD and non-Hodgkin's lymphoma, and the increased frequency of lymphomas in immunosuppressed states, congenital or acquired. This association suggests a link between the development and/or propagation of a neoplastic clone and abnormal immune function. By this reasoning, the prevention or abolition of lymphomas could presumably be altered by restoration of the host immune system.

Rituximab

Rituximab was first approved in 1997 by the Food and Drug Administration (FDA) for the treatment of relapsed/refractory follicular or low-grade NHL. In the registration trial, 166 patients with relapsed, indolent NHL were treated with 375 mg/m [2] of rituximab once a week for 4 weeks [276]. Almost half of the patients demonstrated a response with a complete response rate of 6%. Several other trials confirmed its activity in both treatment-naïve and previously treated cohorts [276–281]. In combination with standard chemotherapeutic regimens, the addition of rituximab has resulted in overall survival prolongation in both DLBCL and FL settings [282–292].

Ofatumumab

Ofatumumab is a human IgGκ monoclonal antibody directed against a novel epitope of the small extracellular loop of CD20 [293]. Its pharmacologic properties suggest a slower disassociation rate and greater stability in vitro in B-cell binding than rituximab. It appears capable of B-cell binding even with low expression of CD20 [294]. In a phase I/II study of 40 patients with relapsed/refractory grade 1 or 2 follicular lymphoma, 4 weekly doses resulted in an overall response rate of over 40% [294]. In a pivotal trial of ofatumumab in patients with CLL refractory to fludarabine and alemtuzumab, an overall response rate of 42% resulted in its approval by the FDA in 2009 for this indication [295]. In a phase III study of 447 adults with treatment naïve CLL who were not considered eligible for treatment with fludarabine, the addition of ofatumumab to chlorambucil vs. chlorambucil alone resulted in higher ORR (82% vs. 69%) and CR rates (12% vs. 1%) [296]. This data led to FDA approval of ofatumumab plus chlorambucil as first line treatment of patients with CLL. Ofatumumab was also studied as monotherapy in phase II trials for relapsed/refractory mantle cell lymphoma and DLBCL with good tolerability but only marginal efficacy [297, 298]. Ofatumumab was also investigated with CHOP in treatment naïve patients with follicular lymphoma. The combination proved to be effective with ORR rates of 90–100% and CR rate of 62% which is comparable to response rates with R-CHOP with a similar safety profile [299].

Obinutuzumab

Obinutuzumab is a glycoengineered type II humanized anti-CD20 mAb that does not require lipid rafts and allows CD20 to remain distributed across the surface of the B cell. Compared to Type I mAbs (rituximab), Type II anti-CD20 mAbs also bind complement at a much lower level which results in higher ADCC and apoptosis [300]. Obinutuzumab in combination with chlorambucil was FDA approved as first line treatment in CLL patient in based off of a randomized

phase III trial of 781 adults which showed superior ORR (78% vs. 65%) and CR (21% vs. 7%) when compared to rituximab plus chlorambucil [301]. In a phase III trial investigating obinutuzumab plus bendamustine (followed by 2y maintenance obinutuzumab) vs. bendamustine alone in patients with rituximab-refractory indolent non-Hodgkin's lymphomas showed almost double the median PFS (29.2 mos vs. 14 mos) for the obinutuzumab plus bendamustine arm with equivalent safety profiles [302].These results led to FDA approval of obinutuzumab plus bendamustine for rituximab-refractory follicular lymphoma in 2016.

Epratuzumab

CD22 is a sialoglycoprotein that is expressed on pre-B and mature B cells that acts as a signaling molecule in cellular adhesion, regulation of B-cell homing, and modulation of B-cell activation [303]. Epratuzumab is a humanized IgG1 mAB targeting CD22 once bound CD22 is internalized and although mechanism of action is unknown, it likely triggers BCR signaling and caspase-dependent apoptosis [304, 305]. Phase I/II studies investigating epraztuzumab as monotherapy in heavily pre-treated indolent and aggressive lymphomas showed that it had antitumoral effects with ORR of 24% in follicular lymphomas and 15% in DLBCL [306, 307]. The combination of epratuzumab and rituximab in relapsed/refractory FL patients resulted in ORR of 54% and CR rate of 24% [308]. In treatment naïve patient with FL, epratuzumab plus rituximab showed a high response rate of 82% with 42.4% CRs and a durage length of response with 60% remaining in remission at 3 years follow-up [309]. In a phase II study of previously untreated patients with CD22+ DLBCL, epratuzumab plus R-CHOP showed a response rate of 96% with 3 year EFS of 70% which compared favorably to traditional R-CHOP chemotherapy although OS was not affected [310].

Alemtuzumab

Alemtuzumab is a humanized monoclonal antibody directed against CD52 and was approved by the FDA for relapsed/refractory CLL/SLL in 2001. This approval was based on the results of a trial reported by Keating and colleagues in which 93 patients with disease refractory to fludarabine and an alkylating agent were treated with alemtuzumab [311]. The median survival was 16 months. It was subsequently approved for treatment-naïve patients in 2007 after the results of a randomized trial in which 292 untreated patients were given alemtuzumab or chlorambucil. Those patients in the alemtuzumab arm benefited with significantly longer progression-free survival at 14.6 months vs. 11.7 months ($p = 0.0001$). The complete response rate was 24% vs. 2% in the alemtuzumab- and chlorambucil-treated patients, respectively [312]. The results of a phase II study of 14 patients with relapsed/refractory peripheral T-cell lymphomas (PTCL) showed an ORR of 36% and three patients achieved CRs although treatment was associated with significant hematologic toxicity and infectious complications [313]. Another phase II study of alemtuzumab in 22 patients with relapsed/refractory mycosis fungoides (MF) and Sezary syndrome (SS) showed a 55% ORR with 32% CRs although again infectious complications were seen in 50% of study patients [314]. Adverse effects secondary to alemtuzumab include cytopenias, infusion reactions, cytomegalovirus reactivation, and other opportunistic infections.

Mogamulizumab

CCR4 is a marker for type 2 helper T cells and regulatory T cells (Treg) expressed on the surface of many patients with CTCL and PTCL [315, 316]. Ligands for CCR4, CCL17, and CCL22 are produced by tumor cells and cells in the tumoral microenvironment attracting CCR4+ Tregs to the tumor where they act to suppress the host immune response thereby allowing tumor immune evasion [317, 318]. Mogalizumab is a defucosylated humanized antibody to CCR4 allowing antibody-dependent cellular cytotoxicity [319] that is currently being investigated in PTCL and CTCL. A phase I/II study of mogalizumab in 41 patients with relapsed/refractory CTCL resulted in ORR of 26.8% with only few grade III/IV toxicities [320]. A phase II study of 38 patients with relapsed/refractory CTCL and PTCL showed mogalizumab achieved an ORR of 35% and CR rate of 14%. Most common adverse effects included cytopenias, infusion reactions, fevers, and skin toxicity [321].

Dacetuzumab and Lucatumumab

Belonging to the tumor necrosis factor receptor super family, CD40 is found on the surface of normal B cells and in most B-cell neoplasms. It appears to elicit ADCC, but not CDC [318]. Dacetuzumab is an agonistic chimeric murine–human monoclonal antibody against CD40. Xenograft studies in lymphoma have suggested a synergistic effect of dacetuzumab with rituximab and gemcitabine [322]. A phase II study of single agent dacetuzumab in relapsed DLBCL showed just modest activity with an ORR of 9% [323]. A phase Ib trial studying dacetuzumab with gemcitabine and rituximab in 33 patients with relapsed/refractory DLBCL observed an ORR of 47% although this is comparable to the efficacy of R-GemOx (rituximab, gemcitabine, and oxaliplatin) in the second line setting [324].

Lucatumumab is a human IgG1 CD40 antagonist that blocks signaling mediated by CD40 and its ligand. In vitro, it demonstrates enhanced ADCC when compared with rituximab [325]. In a Phase IA/II trial of lucatumumab in 111 patients with relapsed/refractory lymphomas, ORR was highest in FL (33.3%) and in marginal zone lymphoma (42.9%) [326].

Immune Checkpoint Blockade

Tumors have developed multiple means by which to evade the host immune response allowing their proliferation. PD-1 (programmed cell death) in an inhibitory transmembrane protein that binds to PD-L1 (programmed cell death ligand). The PD-1/PD-L1 pathway has been revealed as an important regulatory pathway that tumors hijack to directly inhibit apoptosis and cause T-cell exhaustion [327]. PD-1 and PD-L1 are frequently overexpressed in lymphoid malignancies including Hodgkin's lymphoma, FL and DLBCL [328–334]. Blockage of the PD-1/PD-L1 interaction has been shown to reactivatate previously anergic T cells [335]. These discoveries have led to intense ongoing investigation into the utility of targeting this pathway in the management of lymphomas.

Ipilimumab

Ipilimumab is a fully human IgG1κ monoclonal antibody against cytotoxic T-lymphocyte antigen 4 (CTLA-4). CTLA-4 is expressed on the cell surface of CD4+ and CD8+ T-cells and has a higher affinity for the costimulatory receptors CD80 and CD86 on APCs than the T-cell co-stimulatory receptor CD28 thereby terminating CD28 co-stimulation and T-cell activation [336, 337]. Ipilimumab has shown some antitumor effect in relapsed/refractory B-cell lymphomas with a favorable toxicity profile [338–340]. Adverse effects included diarrhea, headache, cytopenias, fatigue, and anorexia. Studies are currently ongoing utilizing ipilimumab in combination with other forms of immunotherapy.

PD-1 Inhibition

Three PD-1 inhibitors have been studied in clinical trials for patients with lymphomas: nivolumab, pembrolizumab, and pidilizumab. Pidilizumab is a humanized IgG monoclonal anti-PD-1 antibody. MD Anderson Cancer Center published results of a phase II trial using pidilizumab plus rituximab for patient with relapse FL. The study found that pidilizumab induced an ORR of 66% with a CR rate of 52% which is favorable when compared to historic controls for single-agent rituximab [341]. Nivolumab is a fully human monoclonal IgG4 antibody to PD-1. In a phase I trial of nivolumab in 23 patients with relapsed/refractory lymphoid malignancies, nivolumab was shown to have good tolerability with ORR highest in DLBCL (36% with 1 CR) and FL (40% with 1 CR) leading to phase II studies in these subtypes [342].

Results from PD-1 blockade in classical Hodgkin's lymphoma have shown promising results. PD-1 is highly upregulated in Reed–Sternberg cells due to gene amplification and EBV infection [331, 334]. As such inhibition of PD-1 has become an attractive strategy in patients with relapsed/refractory classical Hodgkin's lymphoma. A phase 1 study nivolumab in 23 patients with r/r cHL (with 78% post-autologous stem cell transplant and 78% relapsed after brentuximab vedotin) showed an objective response in 87% of participants and an overall acceptable safety profile [343]. This data led to FDA approval of HL for relapse post-autologous stem cell transplant with posttransplant brentuximab vedotin. Pembrolizumab is a humanized IgG4 monoclonal antibody targeting PD-1. In a phase 1b trial, 31 patients with r/r cHL that progressed on or after brentuximab vedotin were given pembrolizumab every 2 weeks. The ORR was 65% with CR rate of 16% in this heavily pre-treated group with 70% of those responses lasting at least 24 months [344]. This data led to breakthrough therapy designation and FDA approval of pembrolizumab in *r/r* cHL.

Brentuximab Vedotin

Brentuximab vedotin (BV) is a antibody-drug conjugate with a CD30 directed antibody linked to auristatin (a microtubule disrupting agent) [345]. BV has been shown in vitro to selectively induce apoptosis in cells bearing the CD30 receptor which is expressed on HRS cells [346]. The FDA initially approved BV in HL relapsed after autologous transplant based on the results of a phase II study in 102 heavily pretreated patients with HL that showed an ORR of 75% and CR rate of 34% with good tolerability. Most common adverse effects were peripheral neuropathy, nausea, fatigue, neutropenia, and diarrhea [347]. A phase 3 trial later evaluated BV as maintenance post-autologous stem cell transplant. The group that received BV as maintenance had an improved median PFS of 42.9 months vs. 24.1 months in the placebo group. These findings allowed for FDA approval of BV as maintenance therapy in the post-autologous stem cell transplant setting [348]. Studies are currently underway attempting to move BV to frontline therapy in combination with doxorubicin, vinblastine, and decarbazine (AVD).

Engineered Chimeric Antigen Receptor T-Cells

Chimeric antigen receptor T cells are autologous T lymphocytes that are genetically modified to express the binding site

of specific antibodies allowing autologous polyclonal T cells to bind a specific tumor associated antigen independent of MHC presentation [349]. The construct usually is composed of a single-chain fragment variable (scFv) fused to the activating intracellular signaling domain of the T-cell receptor, typically the zeta chain [350]. Transduction of the DNA encoding the construct is done ex vivo using retroviral or lentiviral vectors [351–353]. Most clinical experience with CD19 CAR T cells is in acute lymphoblastic leukemia but this is quickly translating to use in other CD19 positive lymphoid malignancies such as non-Hodgkin's lymphoma and CLL.

Researchers at MSKCC initially used CD19 CAR T cells for heavily pre-treated patients with CLL with no preconditioning chemotherapy and found no significant response. The protocol was modified to allow preconditioning fludarabine and cyclophosphamide with a 100% response rate (2 SD, 1 CR, 1 PR) [354, 355]. University of Pennsylvania investigators used the CTL019 CAR T-cell construct in patients with r/r CLL after lymphodepleting chemotherapy and realized a ORR of 57% in a pilot study [356]. In a follow-up phase II trial of 23 patients the ORR was slightly lower at 35% [357]. A team at the NCI tested it's CAR T-cell therapy in a small group of four patients with *r/r* CLL with conditioning fludarabine and cyclophosphamide but added IL-2 with an ORR of 75% [358]. They later dropped the IL-2 from the protocol and realized an ORR of 100% in a second cohort of four patients with three CRs [359].

Data from CAR T-cell therapy in B-cell NHLs is now becoming mature and available. The NCI team evaluated nine patients with DLBCL and indolent B-NHL using fludarabine and cyclophosphamide conditioning followed by their CAR T-cell construct. There was good efficacy in patients with DLBCL with four CRs, two PRs, and one SD. On patient with indolent B-NHL achieved a CR [359]. The UPenn investigators used their CD019 CAR T-cell construct in patients with *r/r* NHL h = who received varying lymphodepleting conditioning chemotherapies. Out of 22 evaluable patients with NHL (DLBCL 13, FL 7, MCL 2) ORR at 3 months was 68% (DLBCL 54%, FL 100% and MCL 50%) [360]. Investigators at the Fred Hutchinson Cancer Center treated 32 patients with r/r B-NHL with conditioning chemotherapy followed by CAR T cells with an objective response of 63%. Patients in this group that received fludarabin and cyclophosphamide had a higher response rate of 72% when compared to those that got cyclophosphamide alone or in combination with etoposide [361].

Despite the positive results from trials employing CAR T cells, their toxicities cannot be ignored. These adverse effects are not usually associated with the dose or timing of infusion but to the expansion or persistence of cells [362, 363]. Most common toxicities are cytokine release syndrome (which can be life-threatening), encephalopathy, and B-cell aplasia. Steroids and IL-6 blocking monoclonal antibody tocilizumab have been utilized for life-threatening CRS with some efficacy [362, 364].

Radioimmunotherapy

Radioisotopes coupled to a monoclonal antibody enable binding of the antibody to the target cell bringing the radioactive portion of the molecule in close proximity to act not only on the cell with the antigen of interest but also on cells that the antibody itself cannot reach. The two most studied radioimmunotherapeutic agents are yttrium-90-ibritumomab tiuxetan (Zevalin) and tositumomab/iodine-131 (Bexxar)

^{90}Y-ibritumomab was approved by the FDA for treatment of relapsed/refractory FL and low-grade NHL. In rituximab-treated patients, the overall repose rate in low-grade lymphomas with ^{90}Y-ibritumomab tiuxetan was 74% with a complete response rate of 15% [365]. In rituximab-naïve patients with relapse/refractory low-grade lymphomas, the overall response rate was 80% in ^{90}Y-ibritumomab tiuxetan-treated patients compared with 56% in the rituximab group. Complete responses were also higher in the radioimmunotherapy arm at 30% compared with 16% in the rituximab-treated patients [366]. In the First Line Indolent Trial (FIT), approximately 400 patients in a complete or partial response after induction therapy (most without rituximab) were randomized to either further therapy with radioimmunotherapy or no treatment [367]. Consolidation with ^{90}Y-ibritumomab tiuxetan resulted in a statistically significant increase in median progression-free survival at 36.5 months vs. 13.3 months without treatment ($p < 0.0001$). Interestingly, 78% of the 101 patients who had a partial response after initial therapy went on to have a complete response after consolidation. ^{90}Y-ibritumomab tiuxetan has been FDA-approved as consolidation treatment for patients with FL who achieve a complete or partial response after induction therapy.

^{90}Y-ibritumomab tiuxetan produces response rates of over 50% in rituximab-untreated patients and 19% in rituximab-exposed patients with refractory DLBCL [368]. A phase III trial investigating ^{90}Y-ibritumomab tiuxetan as maintenance treatment in DLBCL patients in complete remission after R-CHOP has been initiated.

^{131}I–tositumomab has been approved by the FDA for the treatment of CD20 expressing rituximab and chemotherapy refractory lymphoma. In rituximab-untreated patients who have previously been treated with chemotherapy, ^{131}I–tositumomab yields a response rate of 65% with a complete response rate of 20% [369]. In a phase II study of upfront therapy in patients with FL, the overall response rate was 95% and a complete response rate was 75% [370]. The 5-year progression-free survival was 59%. A phase II trial conducted by the Southwest Oncology Group investigated

the use of ^{131}I–tositumomab after six cycles of CHOP in previously untreated FL patients who achieved complete or partial response after chemotherapy [371]. Treatment with ^{131}I–tositumomab was accomplished in 84 of the 90 eligible patients and yielded an overall response rate of 98% and a complete response rate of 74%. Results from a phase III trial comparing CHOP followed by ^{131}I–tositumomab with R-CHOP in treatment-naïve FL patients are awaited.

Delayed myelosuppression appearing approximately 6–8 weeks after treatment remains the most significant adverse event secondary to radioimmunotherapy. Patients with preexisting cytopenias are not candidates for this treatment modality. They include those with absolute neutrophil counts $<1.5 \times 10^3$, platelets <100,000, hemoglobin <9 g/dL, over 25% marrow involvement or prior radiotherapy involving greater than 25% of bone marrow. The possibility of delayed leukemia or myelodysplastic syndromes is debated.

Conclusion

Our understanding and knowledge of the specific immunologic traits of the lymphomas over the past two decades has allowed vast development of immune-directed therapies that have been shown to improve survival in the lymphoid malignancies. The more recent clinical advances in targeting the PD-1/PD-L1 axis and utilizing CAR T-cell therapies has opened the door to a new era in the treatment of lymphomas where we are able to harness ones own immune system to target malignancies. The results of these preliminary clinical trials is encouraging but these therapies are still in their infancy and there is much to learn about the best strategy to utilize them. Still the future for immune therapy of the lymphoid malignancies is promising with multiple treatment options on the horizon.

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42 Cytogenetics of Lymphomas

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Introduction

Cytogenetic analysis relies on the production of banded metaphase chromosomes for analysis but chronic lymphoid malignancies have proved notoriously difficult to karyotype as they have extremely variable rates of growth in culture. The highest number of proliferating cells have been identified in diffuse large B-cell lymphomas (DLBCL) and Burkitt lymphomas (BL) whilst follicular lymphomas (FL) and lymphoplasmacytic lymphomas show decreased proliferation when compared with normal mature B lymphocytes [1]. Therefore, it has been necessary to use a range of tests to determine the genetics of lymphomas, including fluorescence in situ hybridisation (FISH) applied to interphase cells (i-FISH) and metaphase spreads, multicoloured FISH, chromosome comparative genomic hybridisation (CGH) and array-based strategies: array CGH (aCGH) and SNP array (SNP-A) analysis.

The first cytogenetic abnormality to have its genetic secrets unlocked was the 8;14 translocation which characterises Burkitt lymphoma. Researchers identified that the translocation caused two genes, *MYC* on 8q24 and the immunoglobulin heavy chain gene, *1GH* on 14q32, to come together. It is now known that two classes of translocations are found in malignancies. The first type is epitomised by the t(8;14) in Burkitt lymphoma; one gene which is already actively transcribed in the cell type, such as *IGH* in B lymphocytes, is juxtaposed to a gene such as *MYC* which is, by virtue of its resulting proximity to *IGH*, upregulated. Other translocations such as the t(9;22) in chronic myeloid leukaemia form fusion genes with a "new" gene product which incorporates part of the normal genes broken at the sites of translocation. The Burkitt type of translocation is the most common variety observed in chronic lymphoid malignancies as most reciprocal translocations identified in lymphomas involve either the immunoglobulin genes or the T-cell receptor genes in B-cell and T-cell lymphomas, respectively.

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Mature B-Cell Neoplasms

Chronic Lymphocytic Leukaemia/Small Lymphocytic Lymphoma

Chronic lymphocytic leukaemia (CLL), or its non-leukemic counterpart small lymphocytic lymphoma (SLL), is a chronic B-cell lymphoid neoplasm. Cytogenetic studies in CLL have been complicated by the particular difficulty in inducing the malignant cells to divide in culture. Early studies reported normal karyotypes for most cases of CLL, almost certainly because the metaphase spreads obtained in these cases originated from normal cells present in the blood or bone marrow cultures. The addition of B-cell mitogens such as TPA enabled the detection of cytogenetic abnormalities in approximately 35–45% CLL cases. Abnormalities of 11q and 13q were shown to be the most common findings in classical CLL [2] and trisomy 12 to be associated with atypical morphology [2, 3].

In 2006, Dicker and colleagues published a method of culturing CLL/SLL cells using a mixture of CpG oligonucleotide and interleukin-2 enabling abnormal karyotypes to be identified in more than 80% CLLs by conventional cytogenetics [4]. The ability to visualise the karyotype of most CLL cases has confirmed that CLL is characterised by genomic imbalances, with the most common addition being trisomy 12 and the most common deletions being loss of 13q14 and 11q22 [5]. It has also identified a subgroup of patients with a complex aberrant karyotype (≥3 clonal chromosome aberrations) that are more likely to show deletion of 17p, an unmutated *IGHV* status and CD38 expression [5].

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FISH applied to interphase cells has shown genetic abnormalities in most cases studied. A FISH study of 325 CLL cases by Döhner et al. identified abnormalities in 82% cases with deletions of 13q14 (55%) and 11q22 (18%), trisomy 12q (16%) and deletions of 17p13 (7%) and 6q (6%) being the commonest aberrations [6]. None of these aberrations was specific for CLL and so did not provide diagnostic information. However, FISH results correlated with outcome in this cohort. Patients with del(17p) and del(11q) had more advanced disease. Moreover, patients with del(17p) had the shortest median survival and those with del(13q) the longest. A large 2016 study, utilising the CLL Research Consortium database, validated the prognostic utility of the Döhner FISH hierarchy [7]. Median overall survival for patients with del(17p), del(11q), trisomy 12 and del(13q) was 5, 7, 11 and over 12 years, respectively. FISH studies of 18 patients followed for more than 10 years and never requiring treatment showed that none of them had deletions of 17p (*TP53*) or 11q (*ATM*), two had trisomy 12, three had a deletion of 13q. In thirteen patients, none of the studied abnormalities was detected. Thus, in a group of patients with demonstrated indolent disease, the absence of genomic aberrations as demonstrated by FISH correlated with good prognosis [8].

Many studies have shown deletions of 17p13 and 11q22 to be prognostically significant in CLL [6, 7, 9–11]. 11q22 deletions have an association with *ATM* and *BIRC3* mutations, unmutated *IGHV* status, complex karyotype and advanced clinical stage. A study of 118 treatment-naïve patients found that biallelic *ATM* alterations (deletion of one allele with mutation of the second allele) were mutually exclusive of 17p13 deletion and complex karyotype [12]. Biallelic *ATM* abnormalities were an independent predictor of poor overall survival with a hazard ratio similar to that for deletion of 17p13. Deletions of 17p13 have been shown to correlate with the presence of *TP53* mutations, complex cytogenetic abnormalities [13] and with amplification of 2p and deletion of 6q [14]. Del(17p) may be acquired as a secondary event during the course of the disease, particularly in patients with unmutated *IGHV* genes. The proportion of cells with a 17p13 deletion may also be an important prognostic factor. The UKCLL4 trial indicated that 17p deletion in >20% of cells was associated with a dismal complete response rate and a 100% progression rate within 3 years [15]. In the CRC study, patients with del(17p) in <20% nuclei had a substantially longer time to first treatment (TTFT) [7] and Tam et al. reported improved survival in 17p patients with a deletion present in <25% nuclei [16]. However, other groups have shown prognostic significance of 17p deletion using cut-off values of <20% based on the level of aberrant signal patterns identified in normal controls [17, 18] and Rossi et al. reported equivalent outcomes for patients harbouring large and small clones with a mutation in the *TP53* gene [19].

Loss of 13q14 is the commonest finding by FISH. The focus of the deletion has been identified as two microRNAs, *MIR15A* and *MIR16-1,* located in the fourth intron of the *DLEU2* gene [20]. It has been noted that both alleles may be lost and one study of 30 patients suggested that loss of both copies of 13q14 might be a marker for more aggressive disease [21]. Van Dyke et al. grouped 323 patients with a deletion of 13q as a sole abnormality according to locus dosage. TTFT and survival were equivalent for patients who were heterozygous, homozygous or mosaic for 13q deletion, suggesting that it is the presence or absence of a deletion rather than the mono- or biallelic nature of the deletion that dictates clinical behaviour [22]. These findings have been reproduced subsequently in other studies [11, 23]. The number of nuclei found to contain the deletion may have prognostic significance but there is no consensus on the cut-off value, with studies finding statistical significance at >60% [24], >70% [25], and >85% [7] cells, respectively. Accurate mapping of 13q14 deletions using DNA microarray technology has also enabled small lesions (without *RB1* gene deletion) to be distinguished readily from larger deletions (where *RB1* is deleted in addition to *MIR16-1/MIR15A*) [26]. There are conflicting data regarding the impact of larger deletions, with inferior outcomes reported in most [25, 27–29] but not all studies [24].

The significance of abnormalities detected by conventional cytogenetics is an area of active investigation, with a focus on the clinical impact of translocations and karyotypic complexity. In an early study by Mayr et al. translocations, either balanced or more commonly unbalanced, were associated with a significantly shorter TTFT [30]. However, Baliakis and colleagues found that karyotypic complexity but not translocations retained independent prognostic power in multivariate analysis [31]. Complex karyotypes were associated with unmutated *IGHV* status, CD38 expression and abnormalities involving 17p and 11q. Amongst those with complex karyotypes (>3 abnormalities), outcomes were worse in patients with five or more abnormalities. Translocations did have an association with karyotypic complexity, deletion of 17p and the presence of unmutated *IGHV* genes, but this relationship was confined to the unbalanced translocation group [31]. Although the presence of a translocation per se has not been confirmed as an independent prognostic variable, karyotypic complexity has been consistently associated with a poor prognosis [32, 33].

With respect to specific translocation breakpoints, apparently balanced reciprocal translocations involving 13q14 have been observed in CLL but FISH has shown that more than 90% demonstrated a 13q14 deletion at the translocation breakpoint [5]. Moreover, the favourable effect of del(13q) as a sole abnormality detected by FISH is undermined by the presence of translocations, leading to a significantly shorter TTFT [30, 31]. Patients with del(11q) detected by FISH plus translocations also showed significantly shorter treatment free survival when compared with patients with del(11q) without translocations. Most cases of del(17p) detected by FISH were associated with the presence of translocations [30, 34]. A recurrent dicentric unbalanced 17;18 translocation was observed in 16/1213 (1.3%) CLL

cases, resulting in loss of much of 17p and 18p, and associated with early age at diagnosis and disease progression [35].

Reciprocal translocations are uncommon in CLL [5]. Immunoglobulin (IG) translocations have been reported in 5–15% cases [5, 32, 36–38]. Although the majority of these (77%) involve *IGH* at 14q32, variant translocations involving *IGL* (20%) and *IGK* (3%) have also been described. Recurrent partner genes include *BCL2* at 18q21, *BCL3* at 19q13, *MYC* at 8q24, *CDK6* at 7q21, *BCL11A* at 2p16, *BMI1* at 10p12, *FGFR3/MMSET* at 4p16 and *PAX5* at 9p13 [38]. As all of these translocations have been described in a variety of other mature B-cell neoplasms, they lack specificity for CLL.

FISH studies have shown that the presence of an *IGH* translocation predicts a significantly shorter overall survival [32]. The exception to this rule appears to be translocations involving *BCL2* where the prognostic significance is more controversial. The t(14;18) has an association with trisomy 12 and atypical or plasmacytoid morphology in CLL. An adverse impact on prognosis of the t(14;18) was reported in one 2008 study [36], but this has not been confirmed in more recent studies. Put et al. studied 40 patients with CLL and the t(14;18) or a variant translocation. The TTFT in this cohort was 48 months, the estimated median overall survival was 182 months and the t(14;18) was not associated with advanced disease [39]. In a study by Baseggio and colleagues, t(14;18) cases had an intermediate prognosis [40]. In addition, TTFT was statistically superior to that of cases with *BCL3* translocations in the cohort of 64 patients studied by Nguyen-Khac et al. [41] and Davids et al. reported that TTFT was significantly longer in patients with the t(14;18) than in those with other *IGH* translocations [11].

The t(14;19)(q32;q13), has been observed in a variety of B-cell lymphoid malignancies, including CLL, marginal zone lymphoma (MZL) and lymphoplasmacytic lymphoma (LPL). The translocation results in juxtaposition of *BCL3* to *IGH*, resulting in overexpression of BCL3 [42]. The t(14;19) in CLL has been most commonly associated with atypical morphology and immunophenotype, a younger age group with a high proportion of patients diagnosed at less than 40 years of age, the presence of trisomy 12 and deletion of 6q, unmutated *IGHV* genes, rapidly progressive disease and a poor prognosis [41–44]. The t(2;14)(p16;q32) is rare but recurrent in CLL and has an association with atypical or plasmacytoid morphology and unmutated *IGHV* genes [38, 45]. *MYC* translocations are also rare in CLL but have been reported in association with increased prolymphocytes, complex cytogenetic abnormalities, del(11q) or del(17p) and a poor prognosis [46, 47]. *CDK6* translocations are unusual in that they are more likely to involve *IGK* than *IGH,* manifesting as the t(2;7)(p11.2;q21) (see Fig. 42.1); most cases reported to date have had a diagnosis of splenic marginal zone lymphoma or CLL [48, 49].

A recurrent translocation in CLL that does not involve an IG locus is the t(1;6)(p35.3;p25.3) involving the *IRF4/DUSP22* locus at 6p25.3 and an unknown partner gene at 1p35.3 (see Fig. 42.1). This translocation is more common in men than women and has an association with unmutated

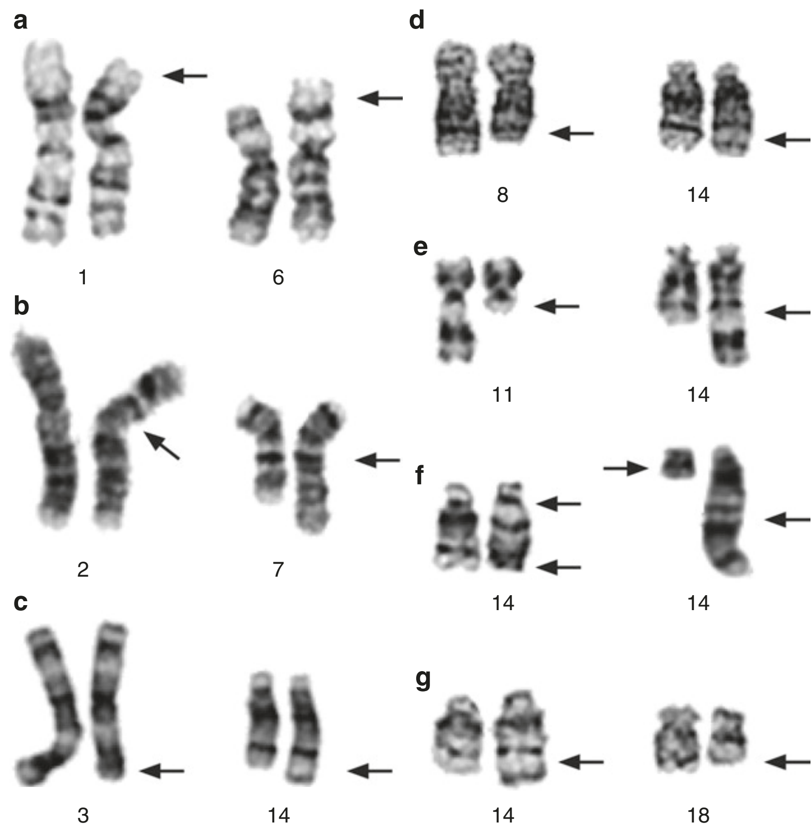

Fig. 42.1 Partial karyotypes of chromosome abnormalities observed in lymphoid malignancies. The abnormal chromosomes are situated on the *right* side of each pair with the normal homologue on the *left*. (**a**) t(1;6)(p35;p25) involving the *IRF4* gene on 6p25 and an unknown partner gene on 1p35; (**b**) t(2;7)(p11.2;q21) involving *IGK* on 2p11.2 and *CDK6* on 7q21; (**c**) t(3;14)(q27;q32) involving the *BCL6* gene on 3q27 and *IGH* gene on 14q32; (**d**) t(8;14)(q24;q32) involving the *MYC* gene on 8q24 and *IGH* gene; (**e**) t(11;14)(q13;q32) involving the *CCND1* gene on 11q13 and the *IGH* gene; (**f**) inversion of chromosome 14 involving the *TRA/TRD* locus on 14q11.2 and the *TCL1A* gene at 14q32 (*left panel*) and the variant t(14;14)(q11.2;q32) (*right panel*); (**g**) t(14;18)(q32;q21) involving the *IGH* gene and the *BCL2* gene on 18q21

IGHV, advanced clinical stage, a requirement for therapy and transformation to high-grade lymphoma [50].

Interstitial deletions of 14q are a recurrent abnormality in CLL. The breakpoints of the del(14)(q24.1q32.33) have been mapped to within the *IGH* locus and in a region of 14q24.1 containing the *ZFP36L1* gene [51]. One gene located within the deleted region is the candidate tumour suppressor gene (TSG) *TRAF3* which has been shown to be homozygously deleted in CLL, or heterozygously deleted with a loss of function mutation in the remaining allele and associated lower expression of TRAF3 mRNA [52]. Deletion of *TRAF3* is one of many genetic events predicted to perturb nuclear factor-KB (NFKB) signalling in mature B-cell lymphomas. FISH studies have also identified patients with deletions of *IGH*. In a series of 291 CLL cases, FISH identified loss of the telomeric 5′ segment of *IGH* (using a 900 kb probe covering the entire variable *IGH* segment) in 82% cases and found the deletion to be associated with good prognosis markers, including del(13q), normal karyotype and lack of CD38 and ZAP-70 expression [37]. However, it appears that these V_H deletions are not oncogenic but rather reflect the physiological events accompanying somatic *V-D-J* assembly [53].

A prognostic scoring system for CLL has been proposed that incorporates age (≥65 years), white blood cell count (≥20 × 10^9/L), unmutated *IGHV* status, *TP53* deletion, the presence of an *IGH* translocation and the number of chromosome aberrations identified by conventional cytogenetics [32]. Using these parameters, patients could be classified into favourable, intermediate and unfavourable risk groups. Genetic factors appear capable of predicting response to therapy. The response to rituximab therapy is significantly lower in CLL patients with del(17)(p13) when compared to patients with other genetic abnormalities identified by FISH [54], possibly due in part to significantly lower CD20 expression [55–57]. Absence of 11q and 17p deletion together with a mutated *IGHV* status is a predictor of favourable response to first-line fludarabine, cyclophosphamide, rituximab (FCR) therapy [58]. Karyotypic complexity was a predictor of event-free and overall survival in relapsed/refractory CLL patients treated with ibrutinib [59]. Complex karyotypes were an independent predictor of outcome after treatment with chlorambucil-based regimens, with the subgroup with karyotypic complexity plus a *TP53* abnormality (deletion or mutation) having a particularly poor prognosis [60].

SNP-A studies have identified the common abnormalities routinely detected by FISH (trisomy 12, deletion of *TP53*, deletion of *ATM* and deletion of 13q14) in the majority of CLL samples [61]. In addition, numerous other copy number changes and regions of copy-neutral loss of heterozygosity (CN-LOH) have been detected. CN-LOH involving the entire length of chromosome 13 and resulting in homozygous deletion of *MIR15A* and *MIR16-1* in the minimally deleted region of 13q14 has been reported [61], together with gain of *MYC* at 8q24, deletion of 8p (*TNFRSF10A/B*) and gain of 2p [62]. The region of gain of 2p in 8–28% CLL cases and up to 39% of CLLs with del(17p) includes the oncogenes, *MYCN, BCL11A* and *REL* [63]. Genomic complexity, as measured by the number of copy number changes identified by DNA microarray platforms, has emerged as an independent risk factor with increased complexity correlating with shortened TTFT [64–67] possibly due in part to the association of del(17p) with higher numbers of copy number aberrations [14, 68]. Array CGH of del(17p) CLLs showed deletions of 18p and/or 20p to be exclusively associated with *TP53* deletion and the higher the percentage of *TP53* deleted cells, the greater the number of additional cytogenetic abnormalities [14]. Regions involved in recurrent abnormalities have also been narrowed by aCGH to a minimal region of 11q encompassing *ATM*, a minimal deletion of 13q encompassing *MIR15A* and *MIR16-1* and a minimal gained region of 2p16 covering the *REL* and *BCL11A* genes [69].

Finally, given the median age of onset of CLL/SLL, the question must be asked as to whether it is cost effective and likely to impact on the choice of therapies to apply a wide battery of tests to older patients. Shanafelt et al. divided a cohort of 2487 CLL patients into those aged <55 years, 55–64, 65–74 and ≥75 years at diagnosis and determined that those aged ≥75 years did not show a reduced life expectancy when compared with age-matched controls and that prognostic testing in this age group had little utility for predicting overall survival [70].

B-Cell Prolymphocytic Leukaemia

B-cell prolymphocytic leukaemia (B-PLL) is a rare neoplasm of B prolymphocytes; it is differentiated from CLL by prolymphocytes representing 55% or more of lymphoid cells in the peripheral blood. Few studies of the cytogenetics of B-PLL have been reported and a retrospective analysis of eight cases of B-prolymphocytic leukaemia (B-PLL) with t(11;14) suggested that these cases represented a splenomegalic form of mantle cell lymphoma (MCL) evolving to leukaemia [71]. Prolymphocytoid transformation of splenic marginal zone lymphoma or splenic diffuse red pulp small B-cell lymphoma can also mimic B-PLL and should be suspected where deletion of 7q is present, especially if there are co-existing deletions of 8p and 17p [72, 73]. Whilst trisomy 12 appears rare, deletions and mutations of *TP53*, and deletions of 11q22.3-q23.1 and 13q14 have been observed in 53%, 39% and 27–55% cases, respectively [74, 75]. The frequency of *TP53* deletions and mutations may explain the aggressive course usually seen in B-PLL. The Burkitt 8;14 translocation and variant 8;22 and 2;8 translocations have

been reported in a handful of cases of B-PLL [47, 76–78] and copy number gain of MYC has also been described [79]. Gene expression analysis comparing B-PLL with CLL showed the two disorders to be biologically distinct with *MYC* one of the genes significantly overexpressed in B-PLL in comparison with CLL [80].

Splenic Marginal Zone Lymphoma

Splenic Marginal Zone Lymphoma (SMZL) is an uncommon low-grade B-cell disorder, which usually presents with a CD5-negative lymphocytosis. Complex chromosomal aberrations are seen in more than 50% cases. The oligonucleotide/IL-2 culture method pioneered by Dicker and colleagues [4] to increase the mitotic index in CLL has also been reported to improve the abnormality rate in SMZL [81].

The commonest abnormalities are gains of 3/3q (15–32%), trisomy 18 (8–12%), trisomy 12 (5–20%) and deletion of 7q32 (21–44%) [82–85]. CN-LOH of 7q has also been reported [86]. The deletion breakpoints at 7q appear heterogeneous with the common area of loss localised using aCGH to 7q32.1-7q32.2 including the *IRF5* gene and a cluster of microRNAs [86–88]. *KLF2* mutations are common in SMZL and deletions of 7q are enriched for in *KLF2* mutant tumours [89]. Arribas and colleagues described a high methylation promoter group in SMZL that was associated with adverse outcomes and had an association with 7q deletion and *NOTCH2* mutation [90]. The presence of two or more chromosomal aberrations may correlate with a shorter survival but the previous reports of an adverse outcome in patients with del(7q) do not appear to be substantiated in larger series [91, 92]. The smallest overlapping region of gain of chromosome 3 was narrowed down to 3q26.33q29 [69]. Rinaldi and colleagues performed DNA microarray studies and identified frequent 7q and 8p deletions in SMZL [72]. Deletions of 8p often co-occurred with 17p deletions and combined deletion of both these regions was associated with a poor prognosis. A second microarray study by Fresquet and colleagues found genomic complexity, deletion of 17p13/*TP53* and gain of 8q/*MYC* to be adverse prognostic factors [86].

Immunoglobulin (*IGH, IGK, IGL*) translocations have been observed in 7%–21% SMZL with a variety of partner chromosomes, resulting in translocations including t(9;14)(p13;q32) (*IGH-PAX5*), t(3;14)(q27;q32) (*IGH-BCL6*), t(14;19)(q32;p13) (*IGH-MUM1*), t(14;19)(q32;q13) (*IGH-BCL3*), t(6;14)(p21;q32) (*IGH-CCND3*) and t(2;7)(p11.2;q21) (*IGK-CDK6*). Numerous other *IG* translocations have been observed but the partner genes have not been identified [92, 93]. Translocations involving *BCL2* and *MALT1* have not been seen in SMZL and although the t(11;14) that is considered characteristic of mantle cell lymphoma has been described in cases of splenic MZBCL, these were reclassified subsequently as mantle cell lymphoma. Most cases of SMZL with *IG* translocations have been shown to contain complex cytogenetic abnormalities in addition to the translocation, but del(7q) and *IG* translocations rarely co-exist [82].

Hairy Cell Leukaemia

With the exception of mutations in the *BRAF* gene, which are present in virtually all cases, recognised recurrent genetic abnormalities are infrequent. Hairy cell leukaemia (HCL) has proven difficult to analyse cytogenetically as the bone marrow aspirate is frequently unsuccessful due to marrow fibrosis. A study using IL4 and anti-CD40 monoclonal antibodies to induce proliferation was able to successfully karyotype 42/43 patients tested but the abnormality rate was only 19% with trisomy of chromosome 5, abnormalities of chromosome 7 and structural rearrangements of 14q being the most frequent abnormalities [94].

A study of splenic tissue from 14 patients with HCL, using CGH and FISH, identified chromosomal gains and losses in only 4/14 cases [95] and a conventional cytogenetics study by Sambani et al. also only found an abnormal karyotype in 8/18 evaluable patients with the commonest abnormality identified being rearrangements of 14q [96]. Further aCGH and SNP arrays also reported few recurrent abnormalities but identified trisomy 5, deletion of 7q and abnormalities of 14q22-32 in a minority of cases [97, 98]. The association of trisomy 5 with HCL was called into question by in the microarray study of Hockley et al. in which trisomy 5 and gain of 5q were more common in HCL-variant than HCL [98]. Nuclear expression of SOX11 has been observed in a subset of HCL associated with upregulation of cyclin D1, but these cases did not contain the t(11;14) that is responsible for overexpression of cyclin D1 in MCL [99].

Lymphoplasmacytic Lymphoma

LPL is a malignant B-cell lymphoma characterised by proliferation of pleomorphic lymphoplasmacytoid lymphocytes and monoclonal IgM production. Mutations involving the *MYD88* gene at 3p22.2 are present in approximately 90% cases of LPL. Accordingly, *MYD88* mutations testing can be useful to distinguish LPL from other B-cell lymphoid neoplasms that may show some plasmacytoid differentiation, such as CLL/SLL, follicular lymphoma and marginal zone lymphoma where *MYD88* mutations are infrequent. LPL has not been shown to harbour the t(11;18), t(1;14) or t(14;18) translocations that are frequently observed in MALT lymphomas and this can be helpful in differentiating between LPL and mucosa-associated lymphoid tissue (MALT) lymphoma [100, 101]. Culture with CpG oligonucleotide and IL-2

achieves abnormality rates up to 80% in LPL [102]. Cytogenetic studies of LPL have shown the most common chromosome abnormalities to be del(6q), observed in 7–55% cases, and trisomy 4; trisomy 12 and *IGH* rearrangements are rarely observed [102, 103]. However, FISH studies found the del(6q) to be an uncommon finding in patients with nodal LPL [104, 105]. An association between trisomy 4 and trisomy 18 has been reported [102, 106]. Trisomy 3 or 3q, resulting from an isochromosome of 3q, has also been associated with B-cell chronic lymphoproliferative disorders with plasmacytoid differentiation [107, 108]. Array CGH applied to LPL cases have also shown del(6q) to be common and an isochromosome 6p pattern where 6q loss is accompanied by gain of 6p is seen in up to 50% of del(6q) cases [109]. CN-LOH of 3p may occur in association with *MYD88* mutation [110]. Frequent regions of gain identified by microarray studies include 3q, 4, 7, 8q, 12q, 18 and Xq. Recurrent regions of loss were 6q, 11q, 13q and 17p [85, 106, 109, 111].

The t(9;14)(p13;q32) involving the *PAX5* and *IGH* genes has been reported in a subset of nodal-based small cell lymphomas with plasmacytoid differentiation [112] but has not been observed in other series of patients with Waldenstrom's macroglobulinemia [113–115]. Moreover, the t(9;14) has been observed in a number of other lymphoma subtypes, including CLL, T-cell rich B-cell lymphomas and diffuse large B-cell lymphomas [116], and so does not appear to be specific to this condition.

Extranodal Marginal Zone Lymphoma of Mucosa-Associated Lymphoid Tissue (MALT Lymphoma)

The MALT lymphomas are common low-grade B-cell lymphomas that arise on a background of chronic inflammation at various mucosal sites. The gastric MALT lymphomas are linked to *Helicobacter pylori* (*H. pylori*) infection and small intestine, skin and ocular adnexal disease have been linked to *Campylobacter jejuni*, *Borrelia burgdorderi* and *Chlamydia psittaci* infections, respectively. MALT lymphomas are characterised by trisomies of chromosomes 3, 12 and/or 18 and a series of reciprocal translocations: t(11;18)(q21;q21), t(1;14)(p22;q32), t(14;18)(q32;q21) and t(3;14)(p14.1;q32) involving *BIRC3-MALT1*, *IGH-BCL10*, *IGH-MALT1* and *IGH-FOXP1,* respectively. The incidence of these translocations varies according to the anatomical site of the lymphoma (see Table 42.1). The t(11;18) is seen predominantly in stomach and lung, the t(14;18) in liver, ocular adnexa/orbit, skin and salivary glands and the trisomies in intestinal and salivary gland lymphomas [117, 118]. The oncogenic products of the t(11;18)(q21;q21), t(1;14)(p22;q32) and t(14;18)(q32;q21) all target the NFKB pathway, which is considered critical to malignant B-cell transformation and lymphoma progression.

Table 42.1 Frequency of translocations in MALT lymphomas

Site of MALT lymphoma	Translocation	Frequency (%)
Stomach	t(11;18)(q21;q21)	57/256 (22%)
	t(1;14)(p11;q32)	8/256 (3%)
	t(14;18)(q32;q21)	1/256 (0.4%)
	t(3;14)(p14.1;q32)	8/208 (4%)
Lung	t(11;18)(q21;q21)	22/62 (42%)
	t(1;14)(p11;q32)	5/62 (8%)
	t(14;18)(q32;q21)	4/62 (6%)
	t(3;14)(p14.1;q32)	0/18 (0%)
Ocular Adnexae	t(11;18)(q21;q21)	8/110 (7%)
	t(1;14)(p11;q32)	0/110 (0%)
	t(14;18)(q32;q21)	14/110 (13%)
	t(3;14)(p14.1;q32)	4/66 (6%)
Salivary gland	t(11;18)(q21;q21)	2/101 (2%)
	t(1;14)(p11;q32)	1/101 (1%)
	t(14;18)(q32;q21)	5/101 (5%)
	t(3;14)(p14.1;q32)	0/60 (0%)
Thyroid	t(11;18)(q21;q21)	1/18 (5.5%)
	t(1;14)(p11;q32)	0/18 (0%)
	t(14;18)(q32;q21)	0/18 (0%)
	t(3;14)(p14.1;q32)	3/13 (23%)
Skin	t(11;18)(q21;q21)	4/108 (4%)
	t(1;14)(p11;q32)	0/108 (0%)
	t(14;18)(q32;q21)	7/108 (6.5%)
	t(3;14)(p14.1;q32)	2/20 (10%)
Liver	t(11;18)(q21;q21)	0/10 (0%)
	t(1;14)(p11;q32)	0/10 (0%)
	t(14;18)(q32;q21)	5/10 (50%)
Intestine	t(11;18)(q21;q21)	3/20 (15%)
	t(1;14)(p11;q32)	2/20 (20%)
	t(14;18)(q32;q21)	0/16 (0%)
	t(3;14)(p14.1;q32)	0/10 (0%)

Frequency data derived from references [118, 124, 131, 132]

In the t(11;18), the apoptosis inhibitor gene *BIRC3* at 11q21 and *MALT1* at 18q21 cause a hybrid gene *BIRC3-MALT1* to form on the derivative chromosome 11. Occasional variant translocations involving 11, 18 and a third chromosome have been described, all resulting in the *BIRC3-MALT1* fusion [119]. MALT lymphomas are known to transform to DLBCL. DLBCL cases with the t(11;18) have only rarely been identified and are predominantly restricted to apparently de novo testicular and breast DLBCL cases [120, 121]. It appears that the t(11;18)-negative lymphomas develop secondary aberrations and are the only MALT lymphomas capable of transformation to DLBCL [122].

A second translocation involving the *MALT1* gene at 18q21 is the t(14;18)(q32;q21), which has an identical conventional cytogenetic appearance to the t(14;18) characteristically seen in follicular lymphoma (FL). The t(14;18) in MALT lymphomas juxtaposes *MALT1* at 18q21, rather than *BCL2*, to the promoter region of *IGH* gene on 14q32 resulting in deregulation of *MALT1* expression [117, 123]. Trisomy 3 and/or trisomy 18 have been observed in association with the t(14;18). Lymphomas with t(14;18) show strong cytoplasmic expression of both MALT1 and

BCL10 [124]. Unlike the indolent MALT lymphomas that are dependent on the continuous presence of *H. pylori*, MALT lymphomas with translocations targeting the *MALT1* gene can be found in cases without *H. pylori* and are resistant to *H. pylori* therapy.

Rare cases of t(14;18) involving the *BCL2* gene with *IGH* rather than *MALT1* have been described in MALT lymphomas. Although it has been suggested that these cases cluster in Hepatitis C-positive patients [125], a case of *IGH-BCL2*-positive MALT lymphoma in a Hepatitis C-negative patient who responded to *H. pylori* eradication therapy has been reported [126].

A less common translocation observed in MALT lymphomas is the t(1;14)(p22;q32) which transfers the *BCL10* gene to chromosome 14 where its expression is inappropriately stimulated by the neighbouring *IGH* enhancer. The upregulated *BCL10* gene product is truncated and loses its weak pro-apoptotic function but retains its ability to activate NFKB. Nuclear *BCL10* expression is also observed frequently in MALT lymphomas that do not exhibit the t(1;14) and has been also associated with the presence of the *BIRC3-MALT1* fusion protein [127]. Rare cases of t(1;2)(p22;p12) have also been reported juxtaposing *BCL10* adjacent to the immunoglobulin kappa (*IGK*) light chain gene [128]. A t(X;14)(p11.4;q32), placing *GRP34* under the transcriptional control of the *IGH* gene regulatory elements, has also been described in one case of parotid MALT lymphoma, two lung MALT lymphomas, one NMZL and one gastric DLBCL [129, 130].

Another translocation involving the *IGH* gene on chromosome 14, t(3;14)(p14.1;q32) has been described in MALT lymphomas, but is also reported in DLBCL and MALT lymphomas with a DLBCL component [131, 132]. The translocation involves the partner gene *FOXP1* on chromosome 3. Trisomy 3 has been observed in conjunction with the t(3;14) and three copies of the *FOXP1* gene identified in 17% MALT lymphomas and 12% MALT lymphomas with DLBCL. However, FOXP1 expression appears independent of the t(3;14) translocation or of copy number changes of *FOXP1*. The t(3;14) does not occur in association with any of the other three primary translocations characteristic of MALT lymphoma [131]. Other rare *IGH* translocations have been identified in single cases of MALT lymphoma, involving the partner genes *CNN3* (1p21.3), *TENM2* (5q34) and *KDM4C* (9p24.1) [133].

The translocations all appeared to occur mutually exclusively in a series of 252 primary MALT lymphomas, with the t(11;18), t(14;18), t(1;14) and trisomies of 3 and/or 18 seen in 13.5%, 10.8%, 1.6% and 42.1% of cases, respectively [118], and the t(11;18) and trisomies of 3 and/or 12 appear unlikely to occur together [134].

Using FISH, trisomies of 3, 12 and 18 were frequently observed in gastric, parotid and thyroid MALT lymphomas but not in most cases of primary breast MALT lymphomas [135]. Thymic MALT lymphomas appear to be most similar to parotid and thyroid tumours with trisomy 3 being the most frequent finding [136]. *MALT1* gene rearrangements have been considered rare in breast MALT lymphomas [135, 137] but were detected in 4/9 cases in one study [138]. Trisomies of 3 and 18 have also been observed in a high proportion of primary intestinal MALT lymphomas [139]. A recurrent deletion of 6q has been observed particularly in ocular adnexal marginal zone lymphomas. It involves a 2.9 Mb region at 6q23.2 to 6q24.1 and the target gene for the deletion is thought to be *TNFAIP3*, an inhibitor of NFKB signalling [140]. However, Kwee et al. detected loss of *TNFAIP3* at similar frequency across a variety of anatomical sites but found that gain of 6p was enriched in ocular adnexal disease and that 8q gain was more frequent in gastric MALT lymphomas [141].

Detection of the common translocations may be via conventional cytogenetics, FISH or RT-PCR. FISH has been applied to cytogenetic suspensions and also to paraffin-embedded tissue. Remstein et al. used two-colour FISH to identify the t(11;18) in nuclei isolated from paraffin-embedded tissue in MALT lymphomas in which the t(11;18) had been previously identified by RT-PCR [134]. FISH showed a fusion signal for the t(11;18) in 92% RT-PCR-positive cases. This study also confirmed that t(11;18) was rarely observed with trisomy 3 or 18.

Eradication of *H. pylori* infection with antibiotics induces complete remission in 70–80% MALT lymphomas but, for gastric MALT lymphomas, the t(11;18) appears to be a clonal marker for resistance to *H. pylori* eradication therapy and antigen independent growth. Liu et al. studied a series of cases with *H. pylori* gastritis, MALT lymphomas and mucosal DLBCL [142]. The *BIRC3-MALT1* fusion transcript was not detected in *H. pylori* gastritis and mucosal DLBCL but was found in 25/72 (35%) of MALT lymphomas at various sites. Its incidence was significantly higher in gastric cases that showed dissemination to lymph nodes or distal sites compared with those confined to the stomach.

Lui et al. extended their studies to 111 patients with *H. pylori*-positive gastric MALT lymphoma who were treated with antibiotics [143]. The t(11;18) was detected in 2/48 complete-regression cases, both of which relapsed subsequently without *H. pylori* reinfection. The translocation was found in 42/63 non-responsive cases, including 26/43 stage 1E. Its presence therefore appeared to be a strong indicator for non-response to antibiotics. Although there have been cases of t(11;18)-positive gastric MALT lymphoma that have achieved durable remissions with *H. pylori* eradication therapy alone [144], the t(11;18) has also been shown to be predictive of resistance to oral alkylating agents with less than 10% durable remission at long-term follow-up [145]. Addition of chlorambucil to rituximab improved response in t(11;18)-positive but not in t(11;18)-negative gastric MALT lymphoma in one prospective, non-randomised study [146].

BCL10 nuclear expression is observed in MALT lymphomas in the presence of the t(1;14) which results in upregulation of BCL10 but is also observed in lymphomas in the absence of translocations that directly target *BCL10*. Normal B-cells express BCL10 in the cytoplasm but MALT lymphoma cells carrying either t(1;14) or t(11;18) show nuclear expression. Such expression has been correlated with advanced disease [142] and resistance to *H. pylori* eradication therapy [147, 148].

Ferreira et al. studied nine MALT lymphomas by aCGH and demonstrated an altered genome in only 33% with no recurrent overlapping regions of imbalance identified [69]. Array CGH of 19 salivary gland MALT lymphomas identified recurrent gains of 1p32-1pter in 43%, 9q33-34 in 84%, 11q11-13 in 42%, chromosome 17 in 58% and 18q21-22 in 42%. Gains of 9q34, 11q13 and 18q21 were frequently concurrent [149, 150]. Deletion of 6q23.3 containing the NFKB inhibitor *TNFAIP3* was observed in MALT lymphomas of the ocular adnexa, salivary gland, thyroid and liver but not lung, stomach or skin. In ocular cases, *TNFAIP3* deletion was associated with a higher proportion of relapses and a shorter relapse-free survival [150].

Array CGH was also used to compare MALT lymphomas with and without the t(11;18). Chromosome gains and losses were considerably more frequent in t(11;18)-negative lymphomas with gains more frequent than losses; these included all or part of chromosomes 3, 12, 18 and 22 in 23%, 19%, 19% and 27%, respectively, and gain at 9q34, the site of the *TRAF2* and *CARD9* genes (interaction partners of BCL10 in NFKB activation) in 42% [151]. The simplicity of t(11;18) cases was confirmed by Braggio et al., with gains of three and eight and loss of 6q again occurring frequently in t(11;18)-negative cases [85]. In those cases without t(11;18), resistance to *H. pylori* therapy was marked by a greater number of genomic imbalances, particularly gain of 1p36.2, chromosome 3 and 18q and loss of 1p36.3 and 7q31-q33 [152]. Moreover, trisomy 3 cases were *H. pylori* negative, t(11;18) negative and resistant to eradication therapy [152]. In t(11;18)-negative gastro-intestinal MALT lymphomas, trisomy of 18q21, including *MALT1*, detected by FISH has been associated with an adverse outcome [153] and the presence of trisomy 18q21 has been strongly correlated with trisomy for 3q27/*BCL6* [154]. Gene expression analysis has revealed that MALT lymphomas harbouring either t(11;18), t(1;14) or t(14;18) show enhanced expression of NFKB target genes whilst translocation-negative cases show active inflammatory and immune responses [155].

Nodal Marginal Zone Lymphoma

Trisomy 3 is recognised as the commonest recurrent cytogenetic finding in nodal MZL. Other frequently observed abnormalities include trisomy 18 and structural abnormalities of chromosome 7 [156, 157]. A cytogenetic study of 103 MZL cases with an abnormal karyotype showed trisomy 3 or an extra copy of the long arm of chromosome 3 to be present in 38/103 (37%) of cases and as a sole abnormality in the stemline of 5/38 cases, but with clonal evolution evident in sidelines [157]. The 3q24 to 3qter segment comprised the most consistently over-represented region. Chromosome 7q deletions were the second most common abnormality, observed in 32/103 patients and in nine as a sole abnormality, occurring as either an interstitial deletion or an unbalanced translocation. The consistently deleted region was 7q33–7q35. Four patients had an unbalanced 3;7 translocation resulting in trisomy of 3q and a 7q deletion. Trisomy 18 or an extra copy of 18q was identified in 29/103 patients, six of whom showed trisomy of 18q as a result of an isochromosome of 18q. Deletion of 6q was seen in 20/103 patients, trisomy 12 in 15 patients and abnormalities of chromosome 8 leading to deletion of 8p were observed in 15 patients, three of whom had an isochromosome of 8q. Lymphoma associated translocations were observed in only 13% patients, including four with the typical mantle cell lymphoma t(11;14), four with t(3;14)(q27;q32) or variants, three with t(9;14)(p13;q32) and two with t(14;19)(q32;q13.1). Comparing their findings with karyotypes of patients with SLL/CLL, MCL and FL, the combination of +3 and del(7q), which was present in 10% MZL, was not observed in any of the other three diagnoses. However, as trisomies of 3, 12 and 18 and deletions of 6q are all recognised abnormalities in lymphoproliferative disorders, they cannot be regarded as specific for MZL. In microarray studies, recurrently gained regions identified in more than one study included 1q, 3q, 12q and 18q; the only recurrent region of loss was 1p [72, 85, 158].

Follicular Lymphoma

FL represent approximately 20–40% of non-Hodgkin's lymphomas. They are generally associated with an indolent clinical course and overall median survival of 8–10 years. The t(14;18)(q32;q21) translocation or its variants, t(2;18)(p12;q21) and t(18;22)(q21;q11.2), are observed in approximately 85% of FL. FL cells are localised to the germinal centre. The characteristic t(14;18)(q32;q21) juxtaposes the *IGH* gene at 14q32 to the *BCL2* gene on 18q21 (see Fig. 42.1). This juxtaposition results in overexpression of the BCL2 protein, which has anti-apoptotic properties. The BCL2 protein is not normally expressed in germinal centre cells.

The variant translocations, t(2;18)(p12;q21) and t(18;22)(q21;q11), involve the *IGK* or *IGL* genes, respectively, rather than *IGH*. There have also been complex translocations described involving a third chromosome breakpoint in addition to 14q32 and 18q21 [159]. The result of all of these rearrangements

appears to be the same, overexpression of BCL2. Despite the anti-apoptotic activity of BCL2, low-grade follicular lymphoma cells carrying the t(14;18) die quickly by apoptosis when cultured in vitro, making cytogenetic studies difficult.

The t(14;18) is observed in most grade 1 and grade 2 FL but in fewer grade 3A and a minority of grade 3B cases. The t(14;18) is also less frequently observed in FL arising in extranodal sites such as skin or testis. Guo et al. [160] identified 69% of FL as "typical" with low histological grade (1 and 2), co-expression of BCL2 and CD10 and containing the t(14;18). They considered this group to be a homogeneous disease entity but the rest to comprise a group of heterogeneous disorders. BCL2 expression may be demonstrated in some cases by immunohistochemistry in the absence of the t(14;18) and there is a case report of a FL with a t(2;18)(q11.2;q21) showing BCL2 overexpression as a result of juxtaposition to the *AFF3* gene at 2q11.2, a non-*IG* translocation [161]. A comparison of gene expression profiles between t(14;18)-positive and -negative FL showed germinal centre B-cell associated signatures in t(14;18)-positive cases whilst activated B-cell like, NFKB proliferation and bystander cell signatures were observed in the t(14;18)-negative cases. In 32% of t(14;18)-negative FL, there was weak or absent CD10 expression and most showed an increased Ki67 proliferation rate. Despite these distinct differences, no survival difference was observed between the two groups [162]. An aCGH study found trisomy 3, gain/amplification of 18q21-qter (including *BCL2*) and loss of 9p21 (*CDKN2A* locus) were significantly more frequent in t(14;18)-negative cases. Most of the t(14;18)-negative cases that showed BCL2 expression by immunohistochemistry exhibited gain/amplification of 18q21-qter and the majority were CD10 negative and MUM1 positive, suggesting that they were derived from late germinal centre B-cells [163].

Additional genetic aberrations are observed in more than 90% FL as, although the t(14;18) is considered the primary genetic event in FL, additional changes are required to produce the fully malignant phenotype. The most frequent secondary numerical aberrations include an extra X chromosome and trisomies of 7, 12 or 12q, 18 or a doubling of the der(18)t(14;18). Structural abnormalities frequently involve chromosomes 1, 6q and 10q22q24 [164–167]. There has been shown to be a correlation of del(1)(p36), del(6q), del(10q) and trisomy 7 with morphological progression but, beyond that, a consistent pattern has not been discerned. M-FISH analysis of a series of FL identified the commonly observed secondary abnormality add(1)(p36) to be an unbalanced 1;17 translocation in approximately one-third of cases [165].

Transformation to high-grade lymphoma occurs in 25–60% cases and is frequently accompanied by the further acquisition of secondary genetic changes. An average number of six chromosome abnormalities were reported in a FL karyotype in addition to the t(14;18) [164]; most of these changes were unbalanced, resulting in gain or loss of chromosomal material. The complexity of secondary abnormalities appears to correlate with grade; higher grade FL contain more abnormalities. A microsatellite study of 9p21 markers in 11 cases of FL that had progressed to DLBCL identified 9p deletions in 73% at transformation, suggesting that del(9p) might be an important secondary event in the histological progression of FL [168]. In another study of 210 patients with FL, 31% of whom transformed to higher grade lymphoma over a 10 year period, an additional X chromosome in the diagnostic karyotype of male patients was the only predictor of adverse outcome [169]. Recent studies have confirmed the importance of del(9)(p21) at transformation and identified other changes including amplification of 2p (including *BCL11A* and *REL*), gains of 6p and 17q21 and *MYC* translocations [170, 171].

Trisomy 7, deletion of 6q and an additional copy of the derivative 18, der(18)t(14;18), were identified as early additional genetic changes with each indicating the first step in different "pathways" of evolution. Deletion of 17p and trisomy 12 were significant negative predictors of overall survival in one large series [166] and del(6q), +5, +19 and +20 were associated with poorer overall survival and del(17p) with poorer event-free survival in another [167]. Deletion at 1p36 was identified in 27/29 t(14;18)-negative FLs and was correlated with predominantly diffuse morphology, often with low clinical stage but large localised inguinal tumours [172]. There appear to be two distinct clinical entities associated with 1p36 abnormalities: nodal-variant paediatric-type FL (PTFL) and t(14;18)-negative FL [173]. CN-LOH of 1p36 in association with mutations in the *TNFRSF14* gene has been reported in 40% cases of IRF4/MUM1-negative, nodal-variant PTFL. In contrast, deletions of 1p36 were observed in t(14;18)-negative FL cases but CN-LOH of 1p36 was rare (9%). PTFL could also be distinguished from t(14;18)-negative FL cases by the low genetic complexity and the relative rarity of mutations involving chromatic modifying genes in the former compared to the latter [173].

Using aCGH, 16 recurrent regional aberrations were identified that were independent predictors of overall survival in multivariate analysis on the following chromosomes:1p-, 1q+, 5p+, 6q-, 8q+, 17p- and 17q+. Two of these, deletions of 1p and 6q, were also shown to be predictors of transformation [174]. Schwaenen and colleagues reported that deletion of 6q was associated with shorter overall survival [175] and O'Shea et al. identified CN-LOH of 1p36 and 16p by SNP-A that predicted reduced survival and disease progression, respectively [176].

A subset of t(14;18)-negative FL contain a t(3;14)(q27;q32) translocation (see Fig. 42.1), resulting in overexpression of *BCL6* on 3q27. *BCL6* rearrangements (3q27) are less common in FL than in DLBCL and are detected in 9–14% of FL cases by conventional cytogenetics.

Paradoxically, the highest levels of *BCL6* expression are found in FL in cases that do not contain 3q27 rearrangements [177]. The t(14;18) is least commonly observed in pure FL 3B subtype, lymphomas composed almost exclusively of centroblasts. It is also rare in the distinct but related group of DLBCL with an associated FL 3B component (DLBCL/FL3B). Translocations involving BCL6 are reportedly rare in pure FL 3B but are relatively frequent in DLCBL/FL3B [178–180]. Analysis of *BCL6* aberrations by FISH in 100 t(14;18)-negative FLs showed that 77 contained a *BCL6* gene rearrangement or translocation and/or gain or amplification of the *BCL6* gene, whilst only 23 had no *BCL6* gene abnormalities. The cases with *BCL6* gene gain/amplification were more likely to show higher grade morphology, higher BCL2 and MUM1 expression and more likely to also show gain of 18q21 (*BCL2*) than other groups [181]. Jardin et al. studied 15 cases of FL with 3q27 translocations. Thirteen of the 15 had a t(3;14)(q27;q32), most were CD10 negative and BCL2 negative but there did not appear to be any significant differences in age, sex, performance status, bone marrow involvement or overall survival, when compared with FL with t(14;18) [177].

Balanced translocations, apart from the t(14;18) and t(3;14), are infrequent but usually represent lymphoma specific changes when present, such as the Burkitt t(8;14) translocation or its variants associated with high-grade transformation. At transformation, 8q24 (*MYC*) translocations are common [169]. Of interest is the observation that FL studied serially showed loss of some cytogenetic aberrations over time that had been present at diagnosis [182]. A small number of FL cases have been reported with a *MYC* translocation present at diagnosis in addition to the t(14;18); this finding may be associated with an aggressive clinical course [182]. However, Ladanyi et al. identified six cases with t(8;14) but no evidence of the t(14;18) in a series of 278 karyotypically abnormal FL. Sequencing showed that the breakpoints did not lie in the regions usually involved in the t(8;14) of Burkitt lymphoma. Moreover, the cases had an indolent course, suggesting that the t(8;14) in FL is not always the same translocation as that seen in high-grade lymphomas [183]. Other rare *IGH* translocations are the t(12;14)(p11.2;q32) placing the *SOX5* gene at 12p11.2 under the transcriptional control of the IGH enhancer [184]. Cases harbouring the cryptic t(6;14)(p25;q32) involving *IRF4*, which has a particular association with grade 3B FL and disease involving Waldeyer's ring, will meet criteria for the provisional entity Large B-cell Lymphoma with IRF4 Rearrangement in the 2016 revision of the WHO Classification [185–187].

It is not generally considered necessary to identify the t(14;18) to make a diagnosis of FL but it may be useful to confirm the diagnosis in small biopsies or with atypical morphology when other tests are inconclusive. Testing for t(14;18) can also be used to monitor therapy, detect recurrent disease and determine the effectiveness of bone marrow purging prior to autologous stem cell harvest. Conventional cytogenetic analysis can detect the t(14;18) but requires fresh tissue with viable dividing cells; in many centres, lymph node biopsies are not routinely submitted for karyotyping.

FISH can be applied to cytogenetic preparations and is also able to detect the t(14;18) in smears, cytospin preparations, touch imprints, frozen tissue and fixed paraffin-embedded tissue. It is best in the identification of the t(14;18) in diagnostic specimens and less effective in detecting residual disease. FISH has a higher sensitivity than PCR as FISH probes span almost all breakpoints whereas standard PCR primer sets cover only a limited number of breakpoint regions. However, PCR analysis is more effective for the detection of minimal residual disease [188].

Mantle Cell Lymphoma

Mantle cell lymphoma (MCL), an aggressive lymphoid tumour, is characterised by distinct clinical, morphological and genetic features. Whilst morphology and immunophenotyping have been widely used to define this entity, the most accurate method of diagnosis is via genetic methods.

The cytogenetic hallmark is the translocation t(11;14)(q13;q32) (see Fig. 42.1), which juxtaposes the cyclin D1 gene (*CCND1,* previously known as *BCL1* and *PRAD1*) with an enhancer sequence of the *IGH* gene at 14q32. Detection of the t(11;14) may be via conventional cytogenetics or FISH using a *CCND1/IGH* dual colour dual fusion translocation probe and FISH may be used on peripheral blood, bone marrow or lymph node samples (see Fig. 42.2). Successful FISH identification of the t(11;14) has also been reported on archived fine needle aspirate cytology slides [189] and paraffin sections [190]. The finding of the t(11;14) is of diagnostic rather than prognostic significance in mantle cell lymphoma. The translocation does not disrupt *CCND1* but results in overexpression and a quantitative increase in normal cyclin D1 protein. Whilst almost half of the 11q13 breakpoints of the t(11;14) in MCL cluster in an 8–10 kb major translocation cluster region, the other 11q13 breakpoints are scattered over more than 100 kb, making PCR-based approaches to identifying the translocation problematic. Comparative analyses of interphase FISH for detection of the t(11;14) with other methods such as conventional cytogenetics, PCR for amplification of the t(11;14) genomic breakpoint, competitive RT-PCR for detection of cyclin D1 transcript overexpression and immunohistochemistry for cyclin D1 protein detection identified FISH as by far the most sensitive method for detecting evidence of the t(11;14) [190–192].

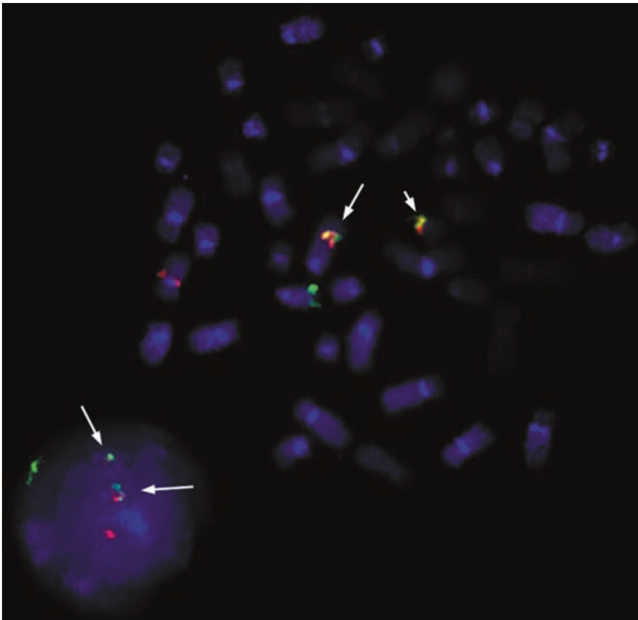

Fig. 42.2 Metaphase spread and interphase cell from a patient with mantle cell lymphoma showing *CCND1/IGH* fusion signals (*arrowed*). The two fusion signals shown on the metaphase spread are located on the der(11)t(11;14) (*short arrow*) and on the der(14)t(11;14) (*long arrow*). The SpectrumOrange™ signal (*CCND1*) is on the normal chromosome 11 and the SpectrumGreen™ signal (*IGH*) on the normal chromosome 14. The interphase cell shows the standard signal pattern of 2 fusions, 1 *red* and 1 *green* that denotes the presence of the balanced t(11;14)(q13;q32) (Probe: LSI *IGH/CCND1* dual colour dual fusion translocation probe, Vysis)

The most common entity from which MCL should be distinguished is CLL as they may have overlapping features, both being CD5-positive small B-cell neoplasms. Reports in the older literature of t(11;14) in CLL almost certainly referred to cases of MCL. A case of splenic lymphoma with histology and flow cytometry results that could not distinguish between splenic marginal zone lymphoma and a mantle cell lymphoma was confirmed as MCL with the demonstration of *CCND1/IGH* fusion signals [193]. Ho et al. identified 28 cases with typical flow MCL phenotype but FISH showed that 9/28 did not contain the t(11;14) and had cytogenetic abnormalities commonly found in CLL. No significant immunophenotypic or morphological differences between t(11;14)-positive and -negative cases could be discerned [194].

The t(11;14) is usually an obvious rearrangement on conventional cytogenetics, although complex translocations masking the t(11;14) have been described, requiring FISH with *IGH* and *CCND1* probes to identify the fusion signals when the t(11;14) in not visible in an abnormal clone. Despite the complex nature of these rearrangements, there are usually abnormalities involving the 11q13 and 14q32 breakpoints, providing a clue as to the underlying genetic rearrangement [195]. However, reports have illustrated the need for FISH despite the finding of an abnormal karyotype with no evidence of any apparent abnormalities of either chromosome 11 or 14. FISH has shown a *CCND1/IGH* fusion signal on a "normal" chromosome 14, apparently caused by an insertion of a sub-microscopic segment of 11q13 into 14q32 [196, 197]. The Spanish Cooperative Group for Hematological Cytogenetics identified 145 cases of MCL at diagnosis with an abnormal karyotype. All contained either a t(11;14), a variant *CCND1* translocation with a three-way translocation involving 11, 14 and a third partner chromosome, a variant translocation involving the *IGL* gene on chromosome 22 (t(11;22)), or a cryptic t(11;14). Nine patients showed the *CCND1/IGH* fusion by FISH only, despite exhibiting an abnormal karyotype [198]. A blastoid variant of MCL did not contain a t(11;14) but had a deletion of 11q and abnormalities of 13p and 21p. Additional M-FISH and locus specific FISH tests identified chromosome 11 and chromosome 14 sequences and *CCND1/IGH* fusion signals on both 13p and 21p [199]. An atypical t(11;14) was identified in 18/103 MCL cases and included patients with duplications of the *CCND1/IGH* fusion, three-way translocations and insertions, emphasising the importance of FISH to confirm unusual translocations [200].

As with most other translocations involving the immunoglobulin heavy chain locus, variant translocations involving one or other of the light chain genes have been reported. Wlodarska et al. reported three cases of the *IGK* variant, t(2;11)(p11;q13), with the 11q13 breakpoint mapped to the 3′ region of *CCND1*, in contrast to the 5′ breakpoint of the t(11;14) [201]. They further postulated that this variant may represent a more indolent form of MCL.

FISH, M-FISH, aCGH and SNP-A have identified extra abnormalities in 80–92% cases [192, 202, 203]. Additional abnormalities with recurrent breakpoints are listed in Table 42.2. There appear to be differences in the patterns of extra abnormalities between those cases shown to have primarily lymph node-based disease and those with peripheral blood disease, with the lymph node-based cases appearing more cytogenetically complex and more likely to show deletions and chromosome loss. Loss of 17p and gain of 3q have been associated with disease progression and to be independent poor prognosis indicators in multivariate analysis [195, 198, 204, 205]. Deletions of *CDKN2A* and *TP53* detected by multiplex ligation dependent probe amplification (MLPA) and PCR were independent predictors of shortened overall survival in the study from the European Mantle Cell Lymphoma Network [206]. The effect of co-existing *CDKN2A* and *TP53* deletions was additive; patients with deletions at both loci had a median overall survival of 1.8 years.

Blastoid and pleomorphic variants appear more likely to contain higher numbers of additional abnormalities, a

Table 42.2 Additional abnormalities in mantle cell lymphoma

Common abnormalities	Recurrent breakpoints	References
+Xq		[209]
−Y		[198, 204]
del(1p)	1p31-32, 1p21-22, 1p36	[198]
+3		[198]
+3q	3q27-28 (*BCL6*)	[198, 204]
+4p	4p12-13	[558]
del(6q)	6q21 6q27	[192, 202]
del(7q)	7q22-35	[205]
del(8p)	8p22, 8p11, 8p21.3 (*TNFRSF10A/B*)	[192]
+8q24	8q24 (*MYC*)	[192]
−9		[198]
del(9p)	9p21 (*CDKN2A/B*)	[204] [192]
del(9q)	9q21	[198]
+10p	10p15	[192]
del(10q)		[204]
+11q	11q13 (*CCND1*)	[202]
del(11q)	11q22.2 (*ATM*), 11q23.3	[192, 202]
+12		[198]
+12q	12q12-13	[192, 204]
−13		[198]
del(13q)	13q13-14 13q21-32 13qter	[192, 204]
+13q	13q31.3 (*MIR17HG*)	[202]
del(15q)	15q23	[192]
+15q	15q23	[192]
del(17p)	17p13.1 (*TP53*), 17p13.3	[192]
−18		[204]
+18q21		[192]
+19p13.3		[205]
del(20p)	20p12.1-12.3	[558]
del(20q)	20q12-q13.2	[558]
del(22q)	22q12.1-12.3, 22q13.31-13.32	[558]

higher frequency of 1p and 17p deletions as well as 10p abnormalities, a higher proliferation index and a shorter survival [205].

The overall number of deletions has been shown to be a significant independent prognostic marker with three or more indicating poor clinical outcome [192]. Deletions of 11q23 and 6q21 have been associated with extranodal disease and deletions of 13q14 and 6q21 were independent predictors of poor outcome in multivariate analysis [207]. MCL studied using metaphase CGH has confirmed the common regions of gain and loss and found gain of Xq and loss of 17p to be associated with a significantly worse prognosis [208, 209]. Deletions of *TP53* have been demonstrated in a high proportion of MCL cases in leukaemic phase [210].

It has been suggested that the ability to identify the abnormal clone by conventional cytogenetics provides some prognostic information. Katzenberger et al. associated complex karyotypes with higher proliferation indices and an inferior prognosis. By conventional cytogenetics, MCL with more than two structural aberrations was characterised by a significantly worse outcome [192]. Complex karyotypes (three or more additional abnormalities) were an independent predictor of inferior survival in the study by Sarkozy et al. [211]. Cohen et al. reported that karyotypic complexity was associated with deletion of 17p. However, karyotypic complexity was not an independent adverse prognostic factor in this case series [212]. Woroniecka et al. correlated the presence of near-tetraploidy and two copies of the t(11;14) with the blastoid variant of MCL and a poor prognosis, the t(11;14) seen as a sole abnormality with an intermediate clinical outcome and a normal result by conventional cytogenetics with a better prognosis [213]. However, the number of cases in this study was small.

Over 80% of MCL cases show classical morphology with less than 15% blastoid or pleomorphic subtypes [192]. Most cases of blastoid MCL have a complex karyotype with three or more chromosomal abnormalities in addition to the t(11;14). Four blastoid MCL cases were characterised by extra *CCND1* copies identified by FISH [214]. Different patterns of additional abnormalities have been distinguished according to the morphological features of the blastoid mantle cells with abnormalities of 13, 18 and 8 commonly seen in "classic" blastoid MCL and abnormalities of 13, 17 and 3 most common in the "pleomorphic" blastoid MCLs. Tumours with prominent nucleoli in the malignant cells have commonly contained chromosome 17 abnormalities [215].

The association of the t(11;14) and one of the Burkitt translocations is a rare but reported observation. Hao et al. reported five cases of MCL with 8q24 abnormalities in addition to the t(11;14), three of which were shown by FISH to have a rearrangement of *MYC* and two an extra copy of *MYC* [216]. All had blastoid morphological features and 4/5 had leukaemic involvement. Vaishampayan et al. also described two cases of blastic transformation of MCL associated with a very short survival with t(8;14) in addition to t(11;14) in one and dup(8q) with an extra copy of *MYC* in the other [217]. Further cases of the association between blastoid MCL and a Burkitt translocation have been reported [218–220] and quantitative PCR showed *MYC* overexpression to be significantly associated with shorter survival in MCL [221]. Another two patients with blastoid variant of MCL and t(11;14) plus *MYC* rearrangements showed Burkitt-like morphology [222]. Thus, MYC overexpression appears to be implicated in blastic transformation of MCL.

A number of cyclin D1-negative MCL cases have been reported. Most of these have been identified as involving either the cyclin D2 locus (*CCND2*) at 12p13 or the cyclin

D3 locus (*CCND3*) at 6p21.2 [223–226]. Translocations involving *CCND2* predominate being observed in approximately 50% of cyclin D1-negative cases. *CCND2* rearrangements result in overexpression of cyclin D2 [227, 228] but immunostaining for cyclin D2 appears non-specific, as it has been observed in most B-cell lymphomas [229]. The t(6;14)(p21.1;q32.3) involving *CCND3* has been observed more often in other mature B-cell malignancies [230]. Despite gene expression analysis revealing upregulation of CCND2 and CCND3 mRNA in cyclin D1-negative cases and showing good correlation with protein expression, interphase FISH has identified translocations or amplification of *CCND2* or *CCND3* in some but not all cases [223, 227]. Of note was the incidence of CNS lymphoma in five of eight cases in one series of cyclin D1-negative MCL [224].

Diffuse Large B-Cell Lymphoma, NOS

Diffuse large B-cell lymphoma (DLBCL), not otherwise specified (NOS) are a group of B-cell neoplasms characterised by proliferation of large cells with a diffuse growth pattern. Estimated to make up approximately 40% of all cases of NHL, DLBCL is one of the commonest types of lymphoma. The WHO entity is defined by inclusion of large B-cell lymphomas that to not meet the criteria for categorisation as particular DLBCL subtypes or other discrete B-cell neoplasms and this makes for considerable clinicopathological heterogeneity within this disease grouping.

Conventional cytogenetics has a high success rate and abnormality rate in DLBCL. Karyotypes in DLBCL are usually complex; greater than 90% of cases have two or more abnormalities. In a study of 363 consecutive cases by Cigudosa et al. 72% of abnormal karyotypes were hyperdiploid, 19% were pseudodiploid and 9% were hypodiploid [231]. Both translocations and copy number changes are prevalent in DLBCL. M-FISH in 12 DLBCL cases showed five with structural abnormalities of chromosome 1, six with loss of 13q and structural abnormalities of chromosomes 7, 18, 6 and 3 in four, five, two and three cases, respectively [232].

The most common translocations in DLBCL involve the *IG* loci at 14q32 (*IGH*), 22q11.2 (*IGL*) and 2p11.2 (*IGK*). A 14q32 breakpoint has been observed in approximately 50% of cases analysed by conventional G-banding [231, 233]. Frequent non-random cytogenetic abnormalities are translocations involving *BCL6*, *BCL2* and *MYC*, which have reported in approximately 20–45%, 20–25% and 5–16% of cases, respectively [231, 233–237]. Less common translocations have also been reported, the *IMMP2L* gene at 7q31, *BCAS2* gene at 1p13 and the *NECTIN2* gene at 19q13 forming novel *IGH* translocations. A non-IG translocation between *MYC* and the tumour suppressor gene *SOCS1* has also been described [238]. FISH studies identified three cases of DLBCL with either a disruption of the *JAK2* gene at 9p24 caused by a t(8;9)(q13;p24) in one case or amplification of JAK2 with add(9)(p24) in two cases [239]. Non-random recurrent copy number changes identified by metaphase CGH include gain of Xq, 2p, 7q, 12p and 18q and loss of 6q and 17p [240]. Array CGH identified gain 1q, 2p, 3p, 3q, 6p, 7p, 7q, 9p, 12q and 18q and Loss 1p13.1-p36.33, 6q16-14, and 17p12-p13.2 [241].

Cell-of-origin (COO) gene expression profiling (GEP) subclassifies DLBCL into germinal centre B-cell type (GC), and activated B-cell type (ABC) or non-GC type [242, 243]. GC-type DLBCLs are thought to arise from germinal centre B-cells, whereas ABC type DLBCLs probably arise from post-germinal centre B-cells arrested during plasmacytic differentiation. Patients with GC-type DLBCL have significantly better responses to chemotherapy than patients with ABC type DLBCL lending weight to the idea that the two groups represent discrete biological entities [243]. However, thus far, translation of GEP COO findings to routine clinical practice has proved challenging. Immunohistochemistry to differentiate GBC and ABC types of DLBCL has shown variable correlation with, but does not precisely reproduce the segregation of patients using gene expression signatures [244]. However, it has become apparent that cytogenetic abnormalities in patients with DLBCL also tend to cluster according to the COO. Using a combination of array CGH and immunohistochemistry, Guo et al. showed differences in the genetic abnormalities in GCB and non-GCB subtypes of DLBCL in a series of 46 patients: GCB was more frequently associated with gain of 7q22.1 and loss of 16q, non-GCB was associated with gains at 11q24.3 and 3q13.2. Within the non-GCB group, those that showed BCL6 positivity by immunohistochemistry, had a higher frequency of gains of 14q23.1 and loss of 6q, whilst the BCL6-negative group were more likely to show gain of 1q, loss of 14q32.13 and a higher number of genomic imbalances overall [245].

The t(14;18)(q32;q21) results in deregulated expression of BCL2 due to juxtaposition of the *BCL2* gene to the immunoglobulin heavy chain enhancer. It is present in over 85% of cases of follicular lymphoma and is also observed in approximately 20% of DLBCL cases. Variant translocations involving *IGK* and *IGL* may also occur. DLBCL cases with *BCL2* translocations are associated with primary nodal disease and the GC type [243]. That is, most patients with a translocation involving *BCL2* are GC type but not all GC-type DLBCL have *BCL2* translocations. It has been proposed that DLBCL cases with a *BCL2* rearrangement may represent transformation of previously unrecognised follicular lymphoma. Frequent secondary abnormalities in t(14;18) karyotypes from DLBCL are trisomy 7 and trisomy 12 [246]. *BCL2* translocation was thought to be indicative of poor prognosis in DLBCL treated with CHOP chemotherapy alone by some

investigators [235, 247]. However with the advent of combination rituximab and multi-agent chemotherapy this no longer appears to be the case [248, 249]. Copy number changes also cluster according to COO. Using aCGH, Tagawa et al. found gain of 1q, 2p, 7q and 12q to be associated with GC-type DLBCL [250]. Gain or amplification of *REL* or 2p has been consistently observed by a number of investigators, [251–254] as has gain of 12q [241, 253–255]. In a comprehensive study combining oligonucleotide aCGH and gene expression profiling in DLBCL, Lenz et al. [254] demonstrated differential use of oncogenic pathways by the GC and ABC types. 12% of patients with GC type and no patients with ABC type DLBCL had amplification of the *MIR17HG* microRNA cluster on chromosome 13. Although this cluster had been linked to DLBCL by other investigators, the study by Lenz et al. was the first to show it was exclusively confined to the GC type. Other novel abnormalities associated with GC-type DLBCL were loss of *PTEN* on chromosome 10 (homozygous loss of *PTEN* was not identified in any ABC type tumours) and deletion of the *TP73* gene on chromosome 1p. Interestingly homozygous *PTEN* deletion was associated with the presence of the t(14;18). *IRF4* (6p25.3) translocations also appear to be a distinct subset of GC-type DLBCL [256] and will comprise the provisional entity LBCL with IRF4 rearrangement in the 2016 revision of the WHO [187].

Meanwhile, translocations involving *BCL6* have been linked to cases of extranodal lymphoma and the ABC (or non-GC) type DLBCL [242, 243]. The most common translocation involving *BCL6* at 3q27 is the t(3;14)(q27;q32) driving BCL6 expression as a result of juxtaposition of the gene to the *IGH* enhancer. Reciprocal translocations between *BCL6* and the light chain loci and other non-immunoglobulin gene partners have also been reported [257–259]. A novel t(3;10)(q27;q11.2) involving the *RASGEF1A* gene was recently reported [260]. In DLBCL cases where the t(3;14) is identified by conventional karyotyping recurrent secondary abnormalities are gain of chromosomes X, 11, 13, and deletion of 6q and 8p23 [246]. The impact of *BCL6* translocation on outcome in DLBCL is unclear. Most studies predate the rituximab era and variously report favourable [234, 261, 262], neutral [263], or adverse [235] prognostic significance. Since CHOP-R has replaced CHOP as first-line therapy for DLBCL it appears that *BCL6* rearrangement has lost any independent prognostic impact [249, 264].

Copy number changes associated with the ABC/non-GC COO by several investigators include trisomy 3/gain of 3q, gain/amplification of 18q, 19q and loss of 6q and 9p21 [243, 247, 250, 254, 255, 265, 266]. 18q amplification results in frequent overexpression of BCL2 mRNA and protein in the APC/non-GC type and was reported to be a poor-prognostic variable in some studies [240, 267] but not in others [241, 253, 254]. Another random, non-recurrent genetic abnormality in the ABC type is mutation of *CARD11* resulting in activation of NFKB signalling, and Lenz et al. reported that *CARD11* mutations and trisomy 3 tended to co-segregate in the small number of patients who had a *CARD11* mutation included in their data set [254]. In this study, ABC DLBCL patients with either trisomy 3, a 9p21 deletion (involving *CDKN2A* locus) or both had significantly worse outcomes than ABC type patients without these abnormalities. Other investigators have also reported patients with 9p21 deletion had adverse outcomes in the CHOP [250] and R-CHOP era [268].

MYC translocations are an important prognostic variable and tend to be more frequent in GC-type DLBCL. Whilst *MYC* translocations are characteristic of Burkitt lymphoma (BL) they are not unique to BL and can be observed in other B-cell lymphomas including CLL, DLBCL, mantle cell lymphoma, follicular lymphoma and plasmablastic lymphoma. They are also identified in high-grade B-cell lymphoma (HGBL) with *MYC* and *BCL2* and/or *BCL6* rearrangements and HGBL NOS, the two new provisional WHO entities having the most overlap with the superseded 2008 BCLU category [187].

The WHO classification restricts BL to tumours with a MYC translocation involving *IG* loci (*IGH* at 14q32, *IGK* at 2p11.2, or *IGL* at 22q11.2) in a simple karyotype. Simple karyotype is defined as no or only a few additional changes or less than six abnormalities detected by aCGH based on the seminal findings of Hummel et al. [269]. To date the guidelines have proved somewhat challenging to interpret in clinical practise as aCGH technology is yet to be widely taken up outside the research or clinical trial setting and the definition of “only a few” is somewhat imprecise. A 2010 study suggested that cytogenetically, distinguishing cases of BL from BCLU (mean additional abnormalities 1.7 and 3.3, respectively) is likely to be more difficult that distinguishing these two entities from DLBCL cases which displayed far more genetic complexity (mean additional abnormalities 21.7) [270]. From these studies it is clear that measures of karyotypic complexity play a role in the diagnostic decision-making algorithm and complement the information obtained from FISH testing in aggressive B-cell lymphomas. Thus an effort should be made to incorporate conventional metaphase karyotyping or molecular karyotyping by aCGH into the work up of lymphomas where there is a high pre-test probability of a *MYC* translocation.

A study measuring the frequency of *MYC*, *BCL6* and *BCL2* rearrangements in a large population of patients with de novo DLBCL using i-FISH found the incidence of MYC rearrangement was 14% [249]. *MYC* rearrangement was rarely present as the sole translocation (only 2% of cases); almost all tumours also had a co-existing 14;18 translocation, a *BCL6* rearrangement or both [249]. These so-called “double-hit” and “triple-hit” lymphomas will comprise the

HGBL with *MYC* and *BCL2* and/or *BCL6* rearrangement group in the 2016 WHO Classification. *MYC* rearrangement per se is associated with a poor prognosis in DLBCL in most published series [266, 271–274]. Double-hit and triple-hit lymphomas are also generally acknowledged to have a poor prognosis [269, 275]. Another large study, using i-FISH to identify *MYC* and *BCL2* gene abnormalities in 327 patients with DLBCL, showed that in addition to patients with double-hit having a poor prognosis, those with GC subtype and isolated *BCL2* rearrangements also had a significantly poorer outcome than those patients without *BCL2* rearrangements [276]. Double-hit DLBCLs are more likely to contain a *BCL2/IG* translocation together with a *MYC* translocation involving a non-*IG* partner gene. This is in contrast to BCLU, which usually present with immunoglobulin genes partnering both *MYC* and *BCL2* [277]. *BCL6/IGH* is much less common than *BCL2/IGH* as the second rearrangement and most of these cases have been found to be triple-hit lymphomas with involvement of *BCL2* as well as *BCL6* and *MYC* [278].

At this stage, it is unclear if the *MYC* translocation partner has prognostic significance. Johnson et al. found that patients with non-immunoglobulin partners (non-*IG-MYC*) tended to have a more favourable outlook than patients with *IG-MYC* translocations [279]. However, in the study by Barrans et al. involving patients treated with R-CHOP, any *MYC* rearrangement as a single parameter remained an independent indicator of poor prognosis [249]. Clipson et al. investigated the impact of *TP53* mutations in a series of 81 DLBCL with *MYC* translocations, and found that *TP53* mutation and *BCL2*, but not *BCL6* translocations had an adverse effect on outcome. In contrast, in those patients without a *MYC* translocation ($n = 153$), *TP53* mutation, *BCL2* and *BCL6* translocations had no effect on outcome [280].

In the study by Seegmiller et al. that excluded non-*IG-MYC* translocations only 1/4 “double hit” lymphomas was classified as a DLBCL; the other 3/4 were BCLU [270]. Despite the caveat that there is some inherent biological heterogeneity in the variably defined “double-hit” DLBCL group, on the whole it does appear that these patients are less likely to respond satisfactorily to R-CHOP regimens [249, 274, 279, 281].

A novel gene fusion between *TBL1XR1* (3q26) and *TP63* (3q28) was seen in 5% cases of DLBCL tested (6 of 115) and 1/81 follicular lymphoma—all DLBCL of the GCB subtype [282]. Also, translocations involving *FOXP1* at 3p13 have been reported in a variety of B-cell lymphomas including DLBCL. The most common is the t(3;14)(p13;q32) juxtaposing *FOXP1* with the *IGH* gene but translocations involving non-immunoglobulin genes have also been observed, generally disrupting the full-length FOXP1 transcript [283].

Gain or amplification of the 11q23-24 region has been described in DLBCL but not well characterised. 11q amplification in the DLBCL cell line KARPAS-422 showed peak amplification to include the region containing the *SIK2/SNF1LK2* gene. The *SIK2* gene was overexpressed in the cell line but overexpression was not detected in primary lymphoma samples [284]. Another study of the gain of 11q observed in DLBCL mapped increase in copy number to the 11q24.3 region in 23% of 166 cases analysed, an area containing the transcription factors *ETS1* and *FLI1*. Both of these genes showed significantly increased expression in the cases with copy number gain and cell line studies showed ETS1 and FLI1 cooperating in sustaining DLBCL proliferation and survival and regulating genes involved in germinal centre differentiation [285].

Chromosome abnormalities were identified in the bone marrow of 192/1585 patients who had had a staging bone marrow for DLBCL. Adverse outcome was associated with two or more cytogenetic abnormalities and specific abnormalities involving 19p13, 12q22-q24, 8q24 and 19q13 [286]. In another conventional cytogenetics study of 110 DLBCL patients with abnormal karyotypes only loss of 17p was associated with an inferior outcome. Loss of 17p was also associated with higher numbers of chromosome abnormalities [287]. High density SNP arrays were used to study 166 DLBCL samples; of the twenty recurrent genetic lesions identified that showed an impact on clinical course, loss of genetic material at 8p23.1 had the most statistically significant negative impact on outcome and was associated with additional aberrations—del(17p) and del(15q) [288]. Clonal heterogeneity, defined as the presence of several sub-clones with different genomic aberrations within a tumour, was identified using array CGH in 55/117 (47%) of DLBCL samples; the average number of copy number aberrations being 7.8 ± 4.3 in those cases without clonal heterogeneity and 12.7 ± 6.1 in those with clonal heterogeneity. Moreover, clonal heterogeneity was associated with gain of 19q13.12-0.43 and loss of 9p21.3 and 17p13.2-11.2. DLBCL with clonal heterogeneity was also shown to have a significantly poorer prognosis [289].

CD5-positive DLBCL has been associated with a poor outcome. Cytogenetic analysis revealed chromosome abnormalities in 10 of 12 cases studied with frequent breakpoints located at 1p13, 3q27, 6q13, 7q32, 14q32, 18q21 and 19q13 [290]. A novel translocation, t(14;16)(q32.33;q24.1) was also identified in a case of CD5+ de novo DLBCL involving the *IRF8* gene on chromosome 16 and *IGH* on chromosome 14 [291].

There appeared to be some differences in the cytogenetic abnormalities seen in germinal centre B-cell-like DLBCL in Asian vs. Caucasian patients with Asian patients showing greater frequencies of *BCL6* rearrangements and gains of 1q and 11q but with lower incidence of t(14;18) [292]. In 231 Chinese patients studied by conventional cytogenetics, FISH and immunohistochemistry, novel patterns of chromosome

abnormalities included gains of chromosomes 5, 13, 14q, 17, 19p, 20, 21p and Y, loss of chromosome 21 and recurrent translocations: t(7;15)(q22;q22), t(3;20)(p24;q13.1) and t(2;3)(q21;q25) [293].

T-Cell/Histiocyte Rich Large B-Cell Lymphoma

T-cell/histocyte rich large B-cell lymphoma (THRLBCL) is a morphological variant of DLBCL characterised by a small number of large CD20-positive B-cells surrounded by an abundant T-cell infiltrate and usually accompanying histiocytes. Morphologically these cases bear some resemblance to cases of nodular lymphocyte predominant Hodgkin lymphoma (NLPHL). Due to the relative paucity of neoplastic cells and contaminating stroma, successful identification of the malignant clone by conventional cytogenetics is problematic. Only two cases with abnormal karyotypes have been reported. Both karyotypes had a near-tetraploid modal number and were complex with numerical and structural abnormalities but no specific abnormality [294, 295]. *MYC*, *BCL2* or *BCL6* rearrangements were not detected. Interestingly, the case reported by De Laval et al. [295] had an isochromosome of 1q, a cytogenetic abnormality that is also frequently observed in NLPHL [296]. Franke et al. performed CGH on DOP-PCR amplified DNA isolated from microdissected CD20-positive cells taken from 17 THRLBCL tumours [297]. Copy number changes were present in all cases. The most frequent changes were gain of Xq, 4q, Xp, 18q21 and loss of 17p. On average THRBCL had less copy number changes than NLPHL cases (4.7 vs. 10.8). However, the authors had noted similar patterns of copy number change in NLPHL tumours previously [298] including gain of 4q that is an unusual finding in other types of lymphoma. Hartmann et al. also compared THRLBCL to NLPHL using array CGH: both lymphomas showed gains of 2p16.1 (involving the *REL* locus) and losses of 2p11.2 and 9p11.2 [299] leading to speculation that THRLBCL and NLPHL may share the same cell of origin.

Primary DLBCL of the CNS

Primary DLBCL of the CNS (CNS DLBCL) is an aggressive B-cell lymphoma restricted to the central nervous system. Investigation of CNS DLBCL by i-FISH has revealed that chromosomal translocations are common in this disorder. They frequently involve the immunoglobulin genes [300, 301]. In a FISH study by Montesinos-Rongen et al. rearrangements of *IGH*, *IGK* and *IGL* were present in 23%, 24% and 36% of cases, respectively [300]. *BCL6* translocations are also frequent, occurring in up to 47% of cases and do not necessarily involve immunoglobulin partners [300–302]. Non-immunoglobulin translocation partners for *BCL6* identified to date include *HIST1H4I*, *HSPCA* and *GAPDH* [302, 303]. The t(8;14), t(11;14) and t(14;18) have not been identified in CNS DLBCL [300] but *MYC* rearrangements by FISH were present in 3% of cases studied by Cady et al. [301]. Gain or amplification of 18q21 is frequent [300, 304]. In addition to 18q21 gain, Weber et al. also identified recurrent loss of 6q and gain of 12q, 22q by CGH [304].

In 2009 Schwindt et al. [305] analysed 19 tumours using a high density SNP-A. Regions of recurrent loss were identified at 3p14.2, 4q35.2, 6p21.32, 6q21, 8q12-12.2, 9p21.3, 10q23.2 and 12p13.2 and there were recurrent copy number gains of 18q21-23, 19q13.31 and 19q13.43 together with gain of the X chromosome and trisomy 12. There was also recurrent CN-LOH at 6p and 9p21.3. Deletion or CN-LOH involving 6p21.32, the site of the HLA Class II genes, was found in 73% of tumours and was the most frequent abnormality detected. Earlier work had also identified deletions at this site as common abnormalities in CNS DLBCL [306–309]. Another nineteen CNS DLBCL samples were subjected to a genome-wide analysis (array CGH and whole exome sequencing) and also showed the most common abnormalities to be deletion of 9p21.3 (*CDKN2A* and *CDKN2B*), deletion of 6p21 (*HLA*), loss of 6q21 (*PRDM1*), 6q23 (*TNFAIP3*) and 6q14.1 (*TMEM30A*). *BCL6* (3q27) and *FOXP1* (3p14) were involved in unbalanced translocations. Recurrent loss/deletion of the *TOX* (8q12) and *PRKCD* (3p21) genes was seen in CNS DLBCL cases but not in systemic DLBCL [289].

A functional consequence proposed to result from loss of HLA class II expression through homozygous deletion or CN-LOH is tumour escape from immune surveillance and absent HLA class II correlates with unfavourable clinical outcomes in some studies [310, 311]. Del(6)(q21), the next most frequent abnormality was present in 53% of cases and is common to DLBCL NOS and other B-cell lymphomas. These findings validate the work of other investigators who has previously reported recurrent loss of 6q, gain of 18q and copy number loss at 9p21.3 in CNS DLBCL using alternative molecular cytogenetic techniques [300, 301, 304, 309, 312, 313].

Gain of 18q21 results in additional copies of the *BCL2* and *MALT1* genes and both have been implicated in disease pathogenesis. However, more recent studies indicating that NFKB pathway activation is ubiquitous in this condition [314] suggest that perturbation of NFKB activity through *MALT1* amplification may be a critical oncogenic event in CNS DLBCL. Similarly, frequent deletion or silencing of *CDKN2A* at 9p21 indicates it is likely to be a critical tumour suppressor gene that must bypassed for CNS DLBCL lymphomagenesis [313, 315, 316].

In the study of 75 immunocompetent patients with CNS DLBCL by Cady et al. del(6q) was an independent predictor

of poor prognosis [301]. This finding was supported by McPhail et al. who used FISH applied to paraffin-embedded sections and identified 14/28 with del(6q) (5/14 homozygous deletions). The deleted group survived less than half the time of the no deletion group (median OS 221 days vs. 516 days) but due to small sample size significance was not achieved ($p = 0.054$) [317].

Loss of 6p22 and homozygous deletion of *CDKN2A* (9p21) in 29 newly diagnosed CNS DLBCL were associated with significantly shorter progression-free and overall survival [318] and in a series of 41 tumours examined by whole exome sequencing, focal deletion or somatic mutations in the *HLA* genes were associated with poor prognosis, and amplification of genes at 7q35 predicted for short progression-free survival [319].

Primary Cutaneous DLBCL, Leg Type

Primary cutaneous DLBCL, leg type (PC LBCL) is a large B-cell lymphoma of the skin often presenting on the leg in elderly women with frequent secondary dissemination. FISH studies in small numbers of patients have indicated that *IGH* translocations are common in this disorder (6/11 patients) [320]. *MYC* and *BCL6* rearrangements have been reported in 6/14 and 2/14 cases, respectively [321]. Copy number changes consistently identified by CGH include gain of 18q and loss of 6q [322].

Dijkman et al. compared 12 cases of PC LBCL to 19 cases of primary cutaneous follicle centre lymphoma (PCFCL) using BAC aCGH and noted distinct patterns of genetic imbalance in the two disorders. Other investigators also noted differences in copy number changes in PCFCL and PC LBCL by CGH [322] supporting their classification as discrete biological entities. In the study by Dijkman and colleagues [323] amplification of 18q was present in 67% of PC LBCL cases and involvement of the *BCL2* and *MALT1* genes was confirmed by i-FISH. Loss of 9p21.3 was identified in 41% of PC LBCL cases and was unique to the PC LBCL subset. Although the number of cases was small, detection of del(9)(p21) by BAC aCGH or *CDKN2A* gene silencing by DNA methylation were associated with poor-prognosis disease in this study. A follow-up study by Senff et al. [324] mapped 9p21 deletions by MLPA and measured *CDKN2A* methylation in a larger series of 64 cases and found deletion to be more common than methylation. Five-year disease specific survival was 43% for cases with a 9p21/*CDKN2A* abnormality as opposed to 70% for those without ($p = 0.06$).

FOXP1 protein is overexpressed in PC LBCL but FISH has not shown the presence of translocations involving the *FOXP1* gene at 3p13. Rather, it has shown low-level copy number gains (3 to 4 copies) of the *FOXP1* gene in 82% of 15 cases studied [325].

EBV-Positive DLBCL of the Elderly

An analysis of copy number alterations in 13 EBV-positive DLBCL identified relatively few genomic alterations compared to 23 EBV-negative DLBCL. The most common aberrations in the EBV+ cases were recurrent gains at 1q23.2-23.3, 1q32.1, 5p15.3, 8q22.3, 8q24.1-24.2 and 9p24.1, and losses at 6q27, 7q11.2 and 7q36.2-36.3. Chromosomal gain at 9p24.1 (*CD274*) was associated with poor overall survival [326]. A case report described the presence of a t(9;14)(p13;q32) in the lymphoma cells of an elderly patient with this disorder. FISH confirmed rearrangement of the *PAX5* gene at 9p13.2 [327].

Primary Mediastinal Large B-Cell Lymphoma

Primary mediastinal large B-cell lymphoma (PMBL) is a B-cell lymphoma presumed to be of thymic B-cell origin arising in the mediastinum without nodal or bone marrow involvement. Compared to DLBCL, it is more frequent in young women and has a more favourable prognosis. PMBL bears a strong resemblance to classical Hodgkin lymphoma (CHL) clinico-pathologically and so-called “grey-zone” lymphoma with features intermediate between the two conditions can pose a diagnostic dilemma.

The most common chromosome abnormalities seen in PMBL are amplifications of the short arm of chromosome 9 and translocations involving the *CIITA* gene on 16p13.13. Early cytogenetic studies identified gain of 9p, 12q and Xq as recurrent cytogenetic abnormalities in PMBL [328–330]. Bentz et al. investigated 43 cases by CGH and observed gains of 9q and Xq in 40% or more of instances [330]. Gain of Xq, 9p21 and 12q14 were confirmed in 87%, 75% and 35% or cases, respectively, using more sensitive i-FISH testing. For other types of B-cell lymphoma assayed by FISH the rate of 9p gain was very low (6/308 cases), identifying 9p gain as a distinctive feature of PMBL. Palanisamy et al. published the results of conventional karyotyping in 17 patients with PMBL. Clonal cytogenetic abnormalities were present in 11/17 (65%) cases. The karyotypes were hyperdiploid or near-tetraploid in the majority of cases with complex numerical and structural abnormalities and no specific aberration. A t(14;18)(q32;q21) was present in two cases and one case had a t(3;14)(q27;q32). Yamamoto et al. also described the presence of *IGH/BCL6* rearrangements located on the der(3)t(3;14) in a case of PMBL [331]. CGH results for 40 PMBL cases were also compared with 91 cases of DLBCL. Gain of 9, 19, X and loss of four were significantly more frequent in the PMBL cohort [332]. Bea et al. compared the chromosome CGH profiles of tumours that had been classified into the ABC and GC subtypes of DLBCL and PMBL by gene expression profiling and

confirmed 9p gain and 2p14-16 gain as distinctive findings in PMBL [255].

BAC aCGH studies in PMBL have been performed by Weesendorf et al. [333] and Kimm et al. [334]. Wessendorf et al. studied 37 cases and reported recurrent gain of 9p24 (68%), 2p15 (51%), 7q22 (32%), 9q34 (32%) and 12q (30%) [333]. Candidate oncogenes *REL* and *BCL11A* were included in a region of high-level amplification on 2p in this study; however the *REL* locus was not part of a larger minimal consensus region of 2p gain. Kimm et al. [334] performed BAC aCGH on samples from 20 patients with PMBL. Fifty-one regions of recurrent genomic imbalance were detected involving 1p, chromosome 2, chromosome 3, 4q, chromosome 5, 6q, 7p, chromosome 8, 9p, 11q, chromosome 12, 17p, chromosome 20, 21q and the X chromosome. Novel regions of gain were 2q13, 3q23, 3q26.1-q27.3, 5q33.3-q34, 7p12.3-p14.1 and 7p22.1-p22.3. Consistent with prior studies the *REL* locus was frequently gained or amplified and there were frequent gains on the X chromosome. *JAK2* copy number was increased in 62% of cases although the region of highest recurrence mapped to 9p13.1-p13.3 and excluded the *JAK2* locus. Recurrent regions of loss were 1p13.1-p13.2, 3p, 4q, 6q and 17p [334].

Weniger and colleagues [335] proceeded to investigate the consistently identified 2p14-p16 copy number gain in greater detail. A FISH probe hybridising to the *BCL11A* locus indicated gain or amplification in 75% or PMBL tumours tested and copy number gain correlated with transcript abundance. Meanwhile *REL* gain amplification was present by FISH in a similar proportion of cases and despite the fact that transcript levels were not consistently increased in cases with increased gene dosage, *REL* copy number increases were associated with nuclear accumulation of the REL protein [336].

In 2008 Lenz et al. [254] used combined oligonucleotide aCGH and gene expression profiling data to compare ABC and GC subtypes of DLBCL with PMBL. The authors identified 30 minimal common regions (MCRs) with subtype distinction. MCRs that occurred with the highest frequency in PMBL were del(6q), 9p amplification or gain, monosomy 10 and 20p gain or amplification. The best discriminator for PMBL was 9p amplification. It was observed in 45% of PMBL but only 11% and 7% of ABC and GCB DLBCL subtypes, respectively. Genes encoding *JAK2* and *PDCD1LG2 (PDL2)* were the most strongly upregulated in the 9p MCR [254, 337]. Meier et al. [338] reported 9p24 gain in 33% of PMBL cases studied by FISH in a tissue microarray study of lymphoma. Translocations involving the programmed death ligand genes *CD274* (*PDL1*) at 9p24.1 and *PDCD1LG2* (*PDL2*) at 9p24.2 are also recurrent in PMBL. Twa et al. used break-apart FISH probes to identify translocations involving these genes in 20% of 125 PMBCL cases with gene rearrangements correlating with elevated transcript levels [339].

Amplification of 9p is also common in classical Hodgkin lymphoma (CHL) and was investigated further using cell lines and primary tumours from patients with CHL and PMBL. Underscoring the importance of oncogenic JAK/STAT signalling in the pathogenesis of these two conditions is the finding that *SOCS1* loss of function mutations resulting in sustained phosphorylation of JAK2 and deregulated STAT activity are present in approximately 50% of PMBL and CHL tumours [340, 341]. With respect to *CD274/PDCD1LG2* amplification in PMBL Green et al. [342] showed *CD274* at 9p24.1 was amplified in 63% (26/41) cases of PMBL and that amplification correlated with transcript abundance. There was also overexpression of *CD274* in tumours from PMBL patients with 9p24.1 amplification using immunohistochemistry. In conclusion, taking into consideration the fact that PDL1 and PDL2 have been implicated in tumorigenesis through reduced immune surveillance, they are also attractive candidate oncogenes within the region of 9p characteristically amplified in the related disorders of PMBL and CHL.

ALK-Positive Large B-Cell Lymphoma

The ALK-positive large B-cell lymphomas represent a rare entity that frequently exhibits plasmablastic morphology. Translocations involving the *ALK* gene on 2p23 occur generally in T-cell or null cell phenotype lymphomas (see below). In 1997, Delsol et al. reported seven patients with large B-cell lymphoma that showed cytoplasmic ALK staining but no evidence of the t(2;5)(p23;q35) that is observed in the majority of ALK-positive T-cell or null cell anaplastic large cell lymphomas [343]. Western blotting of one case showed that the full-length ALK was expressed. Subsequent reports have identified a handful of cases of B-cell lymphomas carrying the t(2;5) and expressing the *NPM-ALK* fusion [344–346]. However, the majority of ALK-positive B-cell lymphomas in both adults and children contain a variant translocation, the t(2;17)(p23;q23), involving the *CLTC* gene at 17q23, which is associated with a distinctive fine granular cytoplasmic localisation of the ALK staining on immunohistochemistry [347–352].

Cryptic *ALK* rearrangements have also been observed, with insertions of *3'ALK* into the *NPM* locus creating a submicroscopic *NPM-ALK* fusion and insertion of *5'SEC31A*, a gene located at 4q21 that had not been previously reported in ALK fusions in lymphoma, upstream of the 3′ end of *ALK* [346]. There have since been two reports of a *SEC31A-ALK* fusion in LBCL—both also involving complex, cryptic rearrangements between 2p23 and 4q21 [353]. The *SEC31A-ALK* fusion was also associated with granular cytoplasmic ALK staining. Translocation partner genes with *ALK* at Xq21 and 12q24.1 have also been reported, both within a single lymphoma in an HIV-positive patient [354].

Burkitt Lymphoma

Burkitt lymphoma (BL) is an aggressive mature B-cell lymphoma of children and adults with a characteristic clinical presentation, morphology, and immunophenotype. Juxtaposition of *MYC* to an immunoglobulin enhancer (*IG-MYC* translocation) resulting in deregulated expression of the MYC proto-oncogene is the hallmark of this condition. In 85% of cases *IGH* at 14q32 is the partner gene, resulting in the reciprocal t(8;14)(q24;q32). In the remaining 15% of cases the translocation involves *IGK* at 2p11.2 or *IGL* at 22q11.2 resulting in the t(2;8)(p11.2;q24) or the t(8;22)(q24;q11.2) variant [355]. Although *MYC* translocations are strongly associated with BL they are not specific for this condition and are encountered in other aggressive B-cell lymphomas, albeit at lower frequency. The distinction is that in BL, there is strong evidence to support *IG-MYC* translocation as the primary or initiating event in lymphomagenesis, whereas in other B-cell disorders *MYC* rearrangement is more likely to occur during evolution of the neoplastic clone. Furthermore, *MYC* translocation is not absolutely required in BL because a small percentage of cases will meet stringent morphological and molecular criteria for BL in the absence of a demonstrable *MYC* translocation. In some cases this may be explained by methodological limitations. For example, screening for *MYC* rearrangement using a FISH break-apart probe may miss both unusual translocations where the breakpoint on 8q is a long way upstream or downstream of the *MYC* locus, and small insertion events. In other cases, evidence suggests that alternative genetic events, such as those leading to downregulation of MIR34B, can mimic the molecular and immunophenotypic consequences of *IG-MYC* translocation [356].

A gene expression profiling study by Dave et al. demonstrated that cases of BL with typical and atypical morphology were interchangeable at a molecular level. Higher expression of *MYC* target genes and germinal centre B-cell genes distinguished cases with a t(8;14) from other lymphomas and, together with reduced expression of genes in the NFKB pathway and MHC Class I genes, reliably distinguished BL from DLBCL with or without the t(8;14) [281]. A contemporaneous study by Hummel et al. [269] involved comprehensive characterisation of mature aggressive B-cell lymphomas using gene expression profiling, BAC/PAC aCGH and i-FISH for *MYC*, *BCL2* and *BCL6* translocations. Consistent with the findings of Dave et al. [281] there was also striking homogeneity of BL cases at a molecular level including cases with atypical morphology.

Using a set of 58 differentially expressed genes, Hummel et al. [269] proceeded to define a molecular gene signature of BL and used it to classify lymphomas into three groups: molecular BL (mBL), non-molecular BL (non-BL) and intermediate. On the basis of i-FISH and aCGH findings the same cohort were also assigned a molecular karyotype. “MYC-simple” was defined as the presence of a *MYC* rearrangement resulting in juxtaposition to an immunoglobulin enhancer, less than six copy number imbalances by aCGH (low genomic complexity score) and the absence of an *IGH-BCL2* fusion or a *BCL6* rearrangement. “MYC-complex” comprised either cases with a non-*IG-MYC* translocation, or an *IG-MYC* translocation together with a high genomic complexity score (six or more genomic imbalances), or a t(14;18) translocation or a *BCL6* rearrangement or any combination of the three. The “MYC-negative” group was all those remaining patients without evidence of a chromosomal rearrangement involving *MYC* at 8q24 by FISH. Eighty-eight percent of patients with mBL had an *IG-MYC* translocation and 91% had evidence of a *MYC* rearrangement, indicating that absence of a *MYC* translocation need not preclude the diagnosis of BL if the clinical presentation, morphology and immunophenotype are otherwise classical.

There was a strong correlation between the mBL gene expression signature and the “MYC-simple” karyotype. Those patients with an intermediate gene expression profile mostly had “MYC-complex” karyotypes, but occasional cases were “MYC-simple” or “MYC-negative.” The majority of cases with a non-mBL gene expression signature had “MYC-negative” karyotypes. Cases with “MYC-simple” karyotypes had significantly better outcomes than the combined “MYC-complex” and “MYC-negative group” (5-year OS 70% vs. 40%, $p = 0.005$). In contrast, in patients with an intermediate or non-mBL gene signature, the presence of a *MYC* rearrangement was associated with a poor prognosis [269].

In order to place the findings of Hummel et al. [269] in the context of conventional metaphase cytogenetic studies, Boerma et al. [355] systematically analysed cases listed in the Mitelman Database of Chromosome Aberrations in Cancer. They defined “true” BL using morphological criteria, presence of an *IG-MYC* translocation and absence of a *BCL2*, *BCL6* or *CCND1* translocation. Variant *IG-MYC* translocations were slightly more common in adults than children, and adults were more likely to have gain of 8q, but in many other respects the karyotypes were very similar, supporting the concept that BL constitutes the same biological entity occurs in children and adults. “True” BL was again characterised by low karyotypic complexity. In 40% of cases an *IG-MYC* translocation was the sole abnormality detected. Of the 205 paediatric cases studied, unbalanced chromosomal abnormalities were present in 60%: one imbalance was present in 33% of cases, two imbalances in 16% and more than two in the remainder. Seegmiller et al. [270] also studied the karyotypic complexity of 34 patients with *IG-MYC* translocations classified according to WHO 2008 criteria. The average number of cytogenetic imbalances was 1.7 in patients with BL, 3.3 in BCLU patients, 6.7 in patients

with plasmablastic lymphoma and 21.7 in DLBCL. Patients with a simple karyotype, defined as less than or equal to two additional cytogenetic abnormalities, had a significantly greater likelihood of having a BL diagnosis. Therefore the investigators proposed that a cytogenetic imbalance score, using a cut-off value of two may be a valuable discriminator between BL and BCLU cases.

Additional secondary abnormalities noted by Boerma et al. [355] in BL karyotypes were gain of 1q, predominantly as the result of a duplication event, trisomy 7 and trisomy 12; losses of 6q, 17p and 13q32-34 were less frequent. Gain of 1q was most often noted as a single additional cytogenetic abnormality but apart from this no clear patterns of secondary abnormality were identified. The pattern of secondary abnormalities in metaphase preparations is in keeping with patterns of genomic imbalance observed by CGH, aCGH and FISH techniques in BL [357]. Lundin et al. studied 20 cases of Burkitt lymphoma by SNP array and identified copy number aberrations in all but one case. Adults contained more abnormalities than children (median 3 vs. 1.5) and recurrent abnormalities included losses of 6q14.1-q22.33, 9p21.3, and 13q14.2-q14.3, gains of 1q23.3-q31.3, chromosome 7, 13q31.3, and CN-LOH for 6p12.2-pter, 9p23-pter, and 17p11.2-pter [358]. Another study compared the additional abnormalities seen in BL in children ($n = 13$) as compared to adults ($n = 11$) and showed that childhood BL was more likely to show 13q31.3q32.1 amplification, gain of 7q32q36 and CN-LOH of 5q23.3 whilst CN-LOH at 17p13 (site of *TP53* gene) and 18q21.3 was only detected in adult BLs [359]. Moreover, relapse or disease progression in 7 BL cases was associated with the acquisition of additional recurrent chromosome aberrations, mainly trisomy 21, gains of 1q and 7q, losses of 6q, 11q, 13q and 17p [360].

Gain of 1q, and 7q and loss of 13q have variously been reported to be associated with inferior outcomes by some investigators [361–363] but there is a dearth of large studies involving uniformly treated patients able to address this question. Nelson et al. [364] studied 90 patients in a multicentre Children's Cancer Group study to assess the prognostic importance of del(13q) and gain of chromosome 7 in BL in children. FISH testing using a MYC break-apart probe, a 13q probe and a chromosome 7 centromere probe were performed on paraffin-embedded tissue (see Fig. 42.3). *MYC* was rearranged in 93% of patients in keeping with results in the earlier study by Hummel et al. [269]. Del(13q) and trisomy 7 were identified in 42% and 10% of cases, respectively. Interestingly the frequency of 13q deletions in this study was far higher by FISH than by conventional cytogenetics suggesting the existence of additional sub-microscopic deletions or the presence of non-proliferating sub-clones not detected by examination of metaphase preparations. Although numbers were small, the presence or absence of a MYC translocation or an additional signal for the chromosome 7 centromere made no difference to outcome; however del(13q) detection by FISH was associated with significantly reduced 5-year overall survival [364]. In another study of 40 adolescents and adult BLs, treated with therapy that included Rituximab, losses of 11q, 13q, 15q or 17p were associated with poor response to treatment, shorter progression-free survival and shorter overall survival [365].

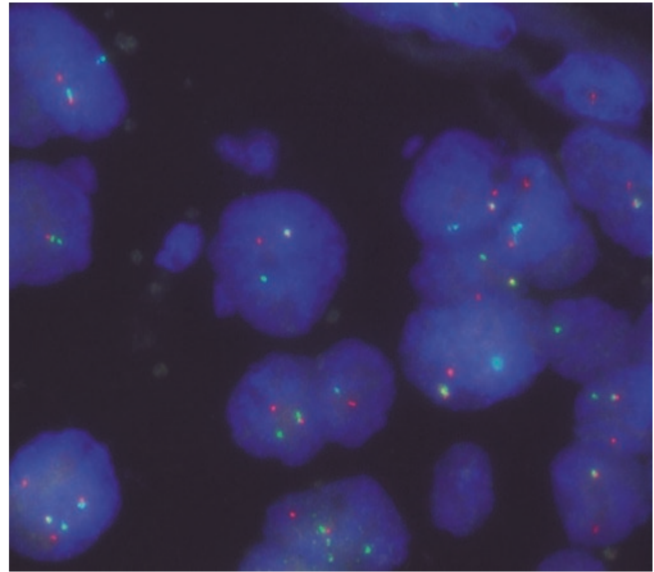

Fig. 42.3 Paraffin-embedded tissue section from the lymph node of a patient with Burkitt lymphoma. A *MYC* break-apart probe (LSI *MYC* dual colour break-apart rearrangement probe, Vysis) shows *MYC* signal rearrangements in most cells, indicating the presence of a translocation disrupting the *MYC* locus at 8q24. The fusion signal indicates an intact *MYC* locus and the red and green signals indicate the presence of a translocation splitting the SpectrumOrange™ labelled *5'MYC* and SpectrumGreen™ *3'MYC* signals apart. This result does not indicate the partner gene involved in the translocation with *MYC*

B-Cell Lymphoma, Unclassifiable, with Features Intermediate between DLBCL and Burkitt Lymphoma (BCLU)

B-cell lymphoma, unclassifiable, with features intermediate between DLBCL and Burkitt lymphoma (BCLU) includes cases of high-grade B-cell lymphoma that do not meet the criteria for Burkitt lymphoma or DLBCL because of intermediate morphology, discordance between morphology and immunophenotype or for other clinical and biological reasons. Patients who meet the criteria for DLBCL who have a *MYC* translocation and patients who meet the criteria for Burkitt lymphoma in the absence of a *MYC* translocation are excluded from this category.

Between 35 and 50 percent of BCLU have a demonstrable *MYC* rearrangement but in contrast to Burkitt lymphoma (BL) many of these involve non-immunoglobulin translocation

partner genes or variant *IG-MYC* translocations involving *IGK* or *IGL* [269, 279]. It may be that in these cases the *MYC* translocation occurs as a secondary event at the time of disease progression, with an *IGH* translocation less likely due to a preceding primary translocation between *IGH* and another oncogene such as *BCL2* at the time of disease initiation [366, 367]. Seegmiller et al. [270] reported karyotypes from patients with BCLU had a mean complexity score of 3.3, whereas BL cases has a score of 1.6, suggesting a cut-off value of two as a useful discriminator in "grey-zone" cases with an *IG-MYC* translocation. Fifteen percent of all BCLU cases have a *BCL2* translocation, and BCLU is enriched for patients with so-called "double-hit" lymphomas (DHL) or "triple-hit" lymphomas, which harbour translocations involving *MYC* and *BCL2* and/or *BCL6* simultaneously [269, 270, 355, 368]. One recurrent translocation involving *BCL6* is the t(2;3)(q21;q27) [369] and two cases have been reported with complex karyotypes, a *BCL2* translocation and a *BCL6/MYC* translocation [370].

The incidence of DHL in a recent large study of 1260 cases of high-grade lymphoma was 4% [279]; approximately two-thirds fitted the criteria for BCLU and most of the remainder were DLBCL. In that subset of DHL cases harbouring an *IG-MYC* translocation rather than a non-*IG-MYC* translocation, studies have consistently found that "double-hit" patients were more likely to be older than patients with BL, have a variant *IG-MYC* translocation, and have greater genetic complexity [269, 270, 355, 368]. Additional non-random, recurrent additional abnormalities identified by conventional cytogenetics in DHL cases were trisomy 7, 8, 11, 12, 18, 20, an additional X chromosome and loss of 3q27-29, 6q, and 15q26 [355].

In the DHL cohort of BCLU and DLBCL cases studied by Johnson et al. the presence of a non-*IG-MYC* translocation was associated with a slightly more favourable outcome, although morphology and IPI remained more powerful predictors of outcome [279]. In other studies, unstratified DHLs whether classified as BCLU or DLBCL are reported to have a poor prognosis [367, 368, 371–373].

Apart from *MYC* and *BCL2* translocations in BCLU, Pienkowska-Gela et al. [374] described four cases with a dup(11)(q23q13) and no evidence of *MYC* translocation. These cases were finally diagnosed as BCLU. Salaverria et al. also identified 19 cases of high-grade B-cell lymphomas with 11q aberrations—interstitial gain of 11q23.2-q23.3 associated with loss of 11q24.1-qter—and showed this 11q abnormality to be frequently associated with morphologic and clinical features of BL despite the lack of a MYC translocation. High-level amplification was observed at 11q23.3, associated with overexpression of genes including *PAFAH1B2*, and deletion of the more terminal region of 11q included loss of the *ETS1* gene, which was also mutated in 4/16 cases tested [375]. Therefore, it appears that duplication of material on 11q represents a novel, recurrent, non-random abnormality in patients in this diagnostic category.

In order to address variable and inconsistent utilisation of the BCLU category, entities that previously fell into this category will be redistributed to the three new provisional entities in the 2016 revision of the WHO: Burkitt-like lymphoma with 11q aberration, high-grade B-cell lymphoma (HGBL) with *MYC* and *BCL2* and/or *BCL6* rearrangements and HGBL NOS [187].

B-Cell Lymphoma, Unclassifiable, with Features Intermediate between DLBCL and Classical Hodgkin Lymphoma

This entity is also known as grey zone lymphoma and appears to have genetic aberrations that are similar to primary mediastinal large B-cell lymphoma (see above). Eberle et al. analysed 27 cases and showed amplification at 2p16.1 (*REL*/*BCL11A* locus) in 33% and aberrations affecting the *JAK2/PDL2* locus at 9p24.1 in 55%. Rearrangement of the *CIITA* locus at 16p13.13 and gains of 8q24 (*MYC*) were observed each in 27% cases [376].

Mature T- and NK-Cell Neoplasms

T-Cell Prolymphocytic Leukaemia

T-cell prolymphocytic leukaemia (T-PLL) is a rare neoplastic disorder of post-thymic T-cells. Clinically the presentation is generally with circulating leukaemia and massive splenomegaly. Often the WCC is greater than 100×10^9/L, which means there is generally little difficulty in obtaining tumour enriched material from blood or bone marrow suitable to culture for karyotyping.

Cytogenetically, the disease is characterised by the inv(14)(q11.2q32) or the t(14;14)(q11.2;q32) which is present in approximately 80% of cases [239, 377–380]. These rearrangements place genes within the *TCL1A* locus under transcriptional control of the enhancer region of the T-cell receptor alpha delta (*TRA/TRD*) locus resulting in deregulated TCL1A expression. Where 14q32 rearrangements are absent, the t(X;14)(q28;q11.2), which translocates *MTCP1* (a *TCL1* homologue) to *TRA/TRD* with similar biological consequences, is usually present [377–379, 381]. Interestingly, patients with the inherited disorder ataxia telangectasia (AT) are predisposed to T-cell malignancy and the inv(14)/t(14;14) or the t(X;14) can be detected in small premalignant clones from these patients suggesting TCL1/MTCP1 deregulation is an early or initiating event during T-cell transformation but is insufficient for development of fully established malignancy [382]. In keeping with the concept that additional genetic

changes are probably required for neoplastic progression, sporadic T-PLL karyotypes are usually complex at diagnosis and typically share a distinctive pattern of secondary cytogenetic abnormalities including abnormalities of chromosomes 6, 8, 11, 12, 17 and 22 [377, 379, 380].

Changes in chromosome 8 are the most common secondary abnormalities and usually result in loss of material on 8p and trisomy for 8q, often as the result of formation of an isochromosome of 8q, an isodicentric chromosome 8p11, or a der(8)t(8;8)(p12;q11.2) [377, 378, 380, 383, 384]. Monosomy or deletion of 11q is the next most frequent abnormality and encompasses the *ATM* gene, which is generally considered the candidate tumour suppressor gene targeted by this deletion in T-PLL [379, 384, 385]. Unsurprisingly, given the association between T-PLL and AT, mutations in *ATM* are also frequent in sporadic cases, such that there is usually biallelic ATM loss of function in this condition even in those patients without 11q22-q23 deletion [385–388]. Deletion of 6q, 12p, monosomy 13/del(13q), monosomy 17/del(17p) and monosomy 22/del(22q) are also seen by conventional karyotyping and/or FISH studies at lower frequencies [377, 379, 389–394]. CGH analysis has identified additional gains on 5p, 6p, 14qter, 21 and 22q12-qter, additional losses on 7q, 9p, 11p, 16q, 17q, and enabled the CDR involved in the del(11q) to be mapped in finer detail [380, 393, 395]. 50K SNP-A analysis of five patients with inv(14)/t(14;14) T-PLL confirmed recurrent gains in 6p and 8q and losses in 6q, 8p and 11q. Furthermore, large novel recurrent areas of loss in 10p and 18p were identified and large regions of CN-LOH on 3q (that were overlapping in two patients), 6p, 9p, 11q and 13q were also reported [393].

JAK3 (19p13) mutations were observed in 11 of 32 patients (34%) with T-PLL; of interest was the finding of 19p13 rearrangements in another 4 of 21 patients lacking a JAK3 mutation [396]. Stengel et al. identified JAK3 mutations in 21% of 51 T-PLL cases; JAK3 mutations had a significant impact on overall survival [384].

T-Cell Large Granular Lymphocytic Leukaemia

This is a T-cell disorder with a distinct clinical presentation, cell morphology and commonly a CD3+ CD8+ CD57+ TCRαβ+ immunophenotype. T-cell receptor gene rearrangements studies are generally used to demonstrate clonality and in most patients the disease follows an indolent clinical course. A large Japanese case series reported normal karyotypes in 33/35 patients; one elderly male patient had a –Y clone and one patient had a structural abnormality of 14q [397]. Similarly Dhodapkar et al. reported a low rate of cytogenetic abnormalities in their series [398]. There are less than 30 published abnormal karyotypes from patients with this condition in the published literature and no consistent pattern of chromosomal derangement is apparent.

Reports are largely confined to conventional cytogenetic analysis. Most karyotypes have a near-diploid modal number and range in complexity from simple numerical aberrations and balanced reciprocal translocations to cases with multiple numerical and structural abnormalities [399, 400]. Man et al. have suggested that monosomy of chromosome 6 or del(6q) may be over-represented in this patient group [399] although deletion of 6q is not unique to this entity.

Chromosomal breakpoints involving the *TRG* and *TRA/TRD* loci at 7p14-p15 and 14q11.2, respectively, are described in patients with this disorder, including some cases of inv(14) or an X;14 translocation involving the *TRA/TRD* locus that are more commonly associated with a diagnosis of T-PLL [400]. Distinguished by a CD3+ CD8+ CD56+ CD57- immunophenotype, a poor prognosis variant of T-cell LGL with a tendency to behave more like aggressive NK cell leukaemia has also been proposed to exist by some investigators [401]. The presence of an isochromosome 7 has been reported in 3/13 cases of aggressive T-cell LGL described to date [401–405]. A case of T-LGLL, γδ variant, was studied by SNP analysis, which revealed CN-LOH at 17q, deletion of 3p21 plus trisomy 5 and monosomies of X and 21 [406].

Chronic Lymphoproliferative Disorder of NK Cells

Chronic lymphoproliferative disorder of NK cells (CLPD-NK) is an indolent disorder in which there is an expanded population of CD3- CD16+ NK cells for greater than 6 months in the absence of an identifiable cause. In retrospective case series of patients subsequently shown to have an indolent clinical course, a normal karyotype at diagnosis is usual [397, 407–409]. T-cell receptors are by definition in the germline configuration, meaning historically there has been a reliance on X-chromosome inactivation to demonstrate clonality in this disorder [408]. Although the presence of a cytogenetic abnormality does not automatically preclude a diagnosis of CLPD-NK, transformation to aggressive LGL leukaemia after 20 months was described in a patient with a mature NK cell lymphocytosis in whom a trisomy 8 clone was present at diagnosis. Evolution of his trisomy 8 clone was coincident with blastic transformation [410].

Aggressive NK Cell Leukaemia

This rare CD2+, surface CD3- (sCD3-), CD56+ NK cell neoplasm with cytoplasmic CD3ε expression is primarily

geographically restricted to Asia, Central and South America, strongly associated with EBV infection and usually involves the peripheral blood, bone marrow, liver and spleen. Although it has a distinct anatomical location and there are differences in patient demography, aggressive NK cell leukaemia (ANKL) appears to share some biological features with extranodal NK/T-cell lymphoma (ENKL). Hence, given their relative scarcity, patients with ENKTL and ANKL tend to be combined in case series where ANKL cases are usually in the minority. Biopsy material is frequently necrotic in this disorder rendering it unsuitable for cytogenetic assays. Furthermore, attempts to obtain abnormal metaphases from involved bone marrow are often thwarted by the low mitotic index of the malignant cells and cellular contamination by non-malignant cells [411] however results from aCGH studies suggest that genomic imbalances occur in the neoplastic cells in most cases.

No unique recurrent abnormalities have been reported in ANKL, although conventional and molecular cytogenetic studies in this disorder are limited. Abnormalities common to other NK cell neoplasms are also found in ANKL including del(6q), del(11q), del(13q), del(17p), trisomy 8, gain of the X chromosome, and isochromosomes of 1q, 6p, 7q and 17q, usually in karyotypes with a near-diploid modal number and three or more abnormalities [411–416]. Spectral karyotyping on a case of ANKL with a complex karyotype identified one translocation involving Xp21 and chromosome 15 together with a unbalanced t(1;8)(q10;p23), demonstrating translocation as a mechanism for gene deregulation in this condition. A translocation with an Xp21 and an 8p23 breakpoint, respectively, were also identified in an additional two cases of ENKL analysed suggesting they may be recurrent breakpoints in these related NK cell disorders [417].

Siu et al. [418] performed conventional cytogenetics and metaphase CGH on patients with ANKL and ENKL. The regions most commonly lost were 6q, 13q, 17p, 9p, 1p, 7p, 10p and 11q and the most frequent gains were observed at 1p, 6p, 11q, 12q, 17q, 19p, 20q and Xp. Del(17p), del(11q), and gain of Xp were more often observed in ANKL than ENKL. Del(6q), del(11q) and del(13q) have been considered the most frequent abnormalities in ANKL [415, 419]. However, a study of 22 Japanese patients confined purely to patients with ANKL failed to identify any instances of del(6q) suggesting that loco-regional differences in the frequency of cytogenetic abnormalities may also be at play [414]. More recently Nakashima et al. [420] performed aCGH on 10 ANKL and 17 ENKL samples. In this series, gain of 1q, together with loss of 7p and 17p were more likely to occur in the ANKL than in ENKL. Notably, loss of 6q and 13q was again infrequent in ANKL in this Japanese study.

Adult T-Cell Leukaemia/Lymphoma

Adult T-cell leukaemia/lymphoma (ATLL) is a T-cell neoplasm encountered in the human T-cell lymphotrophic virus type 1 (HTLV-1) endemic areas of Japan, the Caribbean and Central Africa. ATLL is divided into four clinico-pathological subtypes: acute, lymphomatous, chronic and smouldering. Since chronic infection with HTLV-1 is the causative or initiating event leading to an expanded population of terminally differentiated CD4+, CD25+, CCR4+, FOXP3+ regulatory T-cells, monoclonal integration of HTLV type 1 provirus into cellular DNA is the sine qua non of this disorder. Tumour clonality is readily demonstrable by other molecular means, and cytogenetic techniques are not considered necessary for optimal diagnosis and management of patients in routine clinical practice but may be incorporated into the design of clinical trials as investigational tools [421].

The long latency between HTLV-1 infection in childhood event and disease onset (classically at 50 years of age or older) suggests step-wise acquisition of additional genetic events is required for tumorigenesis. Accordingly, cytogenetic studies revealed karyotypes that were often complex with numerical and structural abnormalities. Frequent gain of chromosomes 3, 7, and 21 and loss of Y, 1p, 3q, 5q, 6q, 7p, 9q, 10p and 13q was seen by conventional karyotyping but no abnormality that was specific for ATLL [422–425]. Abnormality rates were high, with karyotypic abnormalities noted to be more frequent in aggressive than indolent disease [422]. In the study by Itoyama et al. [424] the presence of more than six unrelated abnormalities and abnormalities of 1p, 1q, 2q, 3q, 14q and 17q or loss of 2q, 9p, and 17q were associated with shorter overall survival. Abnormalities at 14q11.2 the site of the *TRA/TRD* locus are reported in approximately 10–15% of patients with ATLL [422, 424] and Haider et al. [426] demonstrated rearrangement of the *TRA/TRD* by FISH in one case of acute ATLL. A CGH study by Tsukasaki et al. [427] of 18 patients with indolent disease and 46 patients with aggressive disease found genomic imbalance was more likely in aggressive ATLL subtypes. Gains of 14q, 7q and 3p were common; regions of loss were less frequent than gains with the most commonly deleted regions being 6q and 13q; 14q and 7q gains were also reported in a smaller independent study by Ariyama et al. [428] involving ATLL cells lines and primary patient samples.

Oshiro et al. [429] compared BAC/PAC aCGH profiles from 17 cases of acute ATLL with 49 cases of lymphoma ATLL. A greater number of genomic imbalances were apparent in the lymphoma type. Recurrent regions of gain common to both types were 3p36, 3q25, 8q24, 9p24, 9q34 and 14q32. The most frequent regions of loss were common to both types were 2q37, 4q21, 6q14, 9p21, 17p13.1 and 19q13. Gain of chromosome 3 or 3p was more common in acute ATLL whereas gain of 7q and loss of 13q were observed

more frequently in lymphoma ATLL. Two-copy loss of 9p21 was frequent consistent with homozygous deletion of *CDKN2A* and *CDKN2B*, which has been associated disease progression from chronic to acute ATLL and with inferior clinical outcomes [430–432].

Deletions/mutations of the *TP53* locus at 17p13.1 have also been observed with disease progression and are estimated to occur in up to 40% of cases of aggressive (acute or lymphomatous) ATLL [423, 433–435]. A study by Tawara et al. [436] of patients with newly diagnosed ATLL found a p53 mutation rate of 17.9% with similar rates in patients with acute (18.6%) and chronic disease (15.4%) whereas *CDKN2A* mutation was over-represented in the acute disease subtype relative to the chronic variant (44.2% vs. 15.4%). Strikingly, *TP53* and *CDKN2A* mutations were mutually exclusive and patients lacking either mutation fared better in terms of overall survival. Furthermore, *TP53* and *CDNK2A* mutation retained their prognostic significance in multivariate analysis.

BCL11B is thought to play a crucial role in T-cell development and BCL11B overexpression has been reported in an acute case of ATLL [429]. Moreover, a t(2;14)(q34;q32) was found in another acute ATLL, forming a *BCL11B-IKZF2* fusion gene [437], and, in a cytogenetic study of 50 ATLL cases where 15% contained an abnormality of 14q32, a t(14;17)(q32;q22-23) was shown to also involve *BCL11B* [438].

Miyata et al. [439] performed BAC/PAC aCGH on DNA derived from skin lesions from patients with cutaneous disease. Gain of 1p, 7q, 18q and loss of 13q were recurrent findings (seen in greater than 20% of cases). Gain of 14q32, observed in 44% and 50% of acute and lymphoma subtypes of ATLL, respectively [429, 440], was only present in 14% of more indolent cutaneous cases. However the study was not geared to determine if 14q32 gain tracked with increased rates of disease progression to more aggressive ATLL. Gain of 1p and loss of 13q were independent predictors of poor prognosis [439].

Extranodal NK/T-Cell Lymphoma Nasal Type

The more common extranodal NK/T-cell lymphoma nasal type (ENK/TL-NT), a tumour involving the upper aerodigestive tract ("nasal" or NK/TL-N) or extranasal sites like skin, soft tissue, the gastro-intestinal tract and testis ("nasal type" or NK/TL-NT), shares many features with aggressive NK cell leukaemia (ANKL), including a strong association with EBV infection and geographical distribution through Asia, Mexico, Central and South America. A CD2+, sCD3-, cytoplasmic CD3ε, CD56+, cytotoxic granule NK cell phenotype not dissimilar to the immunophenotype of ANKL is the norm, but occasional cases have a cytotoxic T-cell phenotype and a monoclonal TCR gene rearrangement.

The inaccessible anatomical location of the tumours and frequent necrosis make it challenging to obtain appropriate tumour material for conventional cytogenetic studies in many cases. Nevertheless there is a high abnormality rate. Recurrent cytogenetic abnormalities observed by conventional karyotyping include del(6q), del(13)q, del(17p), gain of the X chromosome, trisomy 8, isochromosomes of 1q, 6p, 7q, and 17q and rearrangements involving Xp21, 8p23 and 11q23 [411] [412, 413, 417, 425, 441]. CGH studies by a Hong Kong-based group [418] of five cases of ENK/TL-NT revealed loss of X, 6q and 13q, and gain of 1p, 6p, 7q, 11q, 12q, 19, 20 and 22 in at least two cases. Using microsatellite markers in 15 patients the same group showed LOH of 6q, 13q, 17p and 11q in 81%, 67%, 31% and 29% of cases, respectively [419]. In 2001, Ko et al. [442] reported CGH studies in seven Korean patients with ENK/TL-NT and found gain of 2q, 13q, 10q, 21q, 3q, 5q and 17q to be most frequent; the regions most often lost were 1p, 17p, 12q and 13q. Notably only one patient in the Korean cohort [442] had deletion 6q, an extremely frequent finding in the Hong Kong study. Indeed, overall there was very little overlap between the CGH findings of Siu et al. [419] and Ko et al. [442]; however, it is unclear whether this discrepancy between the two studies reflects small sample size, methodological differences, differences in histopathological classification or geographical differences in disease biology.

IN 2005, Nakashima et al. [420] compared 10 cases of ANKL with 17 cases of ENK/TL-NT by aCGH. Gain of 2q, and loss of 6q16.1-q21, 11q22.3-q23.3, 5p14.1-14.3, 5q34-35.3, 1p36.23-p36.3, 2p16.1-16.3, 4q12 and 4q31.3-q32.1 were the strongest positive predictors for a diagnosis of ENK/TL-NT as opposed to a diagnosis of ANKL. Array CGH studies of seven patients with NK cell malignancy were reported by Iqbal et al. [443]. The pattern of genomic imbalances observed closely approximated the findings of Nakashima et al. [420] with some overlap with the data set of Siu et al. [419] including gain of 1q, 2q, 6p, 7q, 17q and 20q and loss of 6q, 11q, 13q, and 17p. Huang et al. [444] analysed nine cases of ENK/TL-NT by BAC aCGH. Recurrent areas of copy number gain were 1q21-q44, 4p16, 6p25-p11.1, 6q11.1-q14, 6q27, 7p11.2-q34, 7q11.2-q34, 7q35-q36, 8p23.3, 9q34, 10p15-p14, 11p15, 16p13.3, 17q12 and 22q11.21; recurrent areas of loss were 6q16-q25, 8p23-p22, 11q24-25 and 17p13.3. Interestingly, regional gain of 1q, del(6q) and del(17p) which were recurrent in this cohort were also found by Iqbal et al. [443]. Berti et al. [445] compared aCGH tumour profiles from three patients with primary cutaneous ENK/TL-NT to a skin tumour from one patient with disseminated ENK/TL-NT. Gain of 1q, 7q and deletion of 17p were seen in at least 2/3 patients with primary cutaneous disease and loss of 6q in one patient. The skin tumour from the patient with ENK/TL-NT also had gain of 7q along with loss of 9p and 12p that was not observed in

the primary cutaneous tumours. An array CGH study of 13 cases of ENK/TL-NT showed the most common abnormalities to be loss of 15q24 and 19q13.3 regions (both seen in 9/13, 69%). Moreover, loss of 8p11 was associated with a significantly shortened survival [446].

Enteropathy-Associated T-Cell Lymphoma

Enteropathy-associated T-cell lymphoma (EATL) is a highly aggressive primary T-cell neoplasm arising in the gastrointestinal tract. Monoclonal T-cell receptor gene rearrangements are present. Nonmonomorphic or type I EATL (known as enterophathy-associated TCL in the 2016 revision of the WHO [187]) is a disease of Western countries that has a strong association with coeliac disease. Tumour cells usually display surface expression of CD8 but not CD56 and have pleomorphic, anaplastic or immunoblastic morphology. In contrast EATL type II (recast as monomorphic epitheliotropic intestinal TCL (MEITL) in the 2016 revision of the WHO [187]) has a broader geographical distribution and an association with coeliac disease is not proven. Type II tumours have a monomorphic appearance and frequently co-express CD8 and CD56. Clonal cytogenetic abnormalities have been reported in only a small number of patients with EATL using conventional karyotyping without any consistent pattern of derangement being identified [447].

In 2002, Zettl et al. [448] analysed DNA from 38 formalin-fixed paraffin-embedded tumours or fresh-frozen tumours by CGH. Copy number aberrations were present in 87% of tumours studied. Strikingly gain of 9q was observed in 58% of cases, with particularly localised gain at 9q33-34 confirmed by FISH testing. Other frequently gained regions were 7q (24%), 5q (18%) and 1q (16%). 8p and 13q were deleted in 24% of cases, 9p in 18% and 16q in 8%. Clinical outcomes were worse in patients with tumours that carried more than three copy number aberrations. Interestingly, partial trisomy of 1q has been identified in a high proportion (6/7) patients with refractory coeliac sprue, a preneoplastic or precursor lesion to nonmonomorphic or type I EATL suggesting that deregulation of candidate genes on 1q is an early event in the transformation of intestinal T-cells [449]. FISH studies by Leich et al. [450] in 2007 identified gain of 7q in 14/51 EATLs tested. As this included ten cases that had been studied by CGH previously [448], the FISH patterns could be interpreted in conjunction with imbalances detected by CGH. In at least two cases, and up to six cases, the abnormalities detected were consistent with isochromosome formation as the mechanism for 7q gain in this disorder.

Deleeuw et al. [451] published the results of higher resolution aCGH studies on thirty samples in 2007, confirming and further refining the copy number imbalances detected by CGH. The 9q gain resolved into discrete regions of alteration at 9q31.3-q33.2, 9q33.2-q33.3 and 9q34.11-qter. There was copy number gain at 9q33.2-q33.3 in 67% of cases. The 5q gain was mapped to 5q34-35.2. The 1q gain was reconfigured to three regions: 1q23.1-23.3, 1q25.3-31.2 and 1q32.2-41. The most distal region on 1q was most frequently involved (47% of cases) and was highly amplified in one case. Gains on 7q were also found to be segmental and complex with gain of 7q33-34 being the most common. 36% of cases had loss of 8p22-23.2 making this the most frequently lost region. The 16q deletion was mapped to 16q12.1. The 9p copy number loss spanned 9p21.2-p21.3, a region that encompasses the *CDKN2A* and *CDKN2B* genes. The investigators noted that 9q gain and 16q loss were almost mutually exclusive and one or other abnormality was present in virtually all cases studies. Gain of the *MYC* oncogene at 8q24 was highly enriched for in samples from the monomorphic type II EATL patient cohort. Principal components analysis using all regions of alterations showed increased copy number at 8q24 and 1q gain were the major factors contributing to separation of nonmonomorphic/type I EATL from type II EATL. In summary, gain of 9q31 or deletion of 16q21 occur in greater than 80% of cases of both EATL (type I) and type II EATL and appear to be unifying or characteristic of the condition as a whole. Furthermore, gain of 1q32.2-q41 (73% vs. 20%) and 5q34-35.2 (80% vs. 20%) are strongly overrepresented in type I EATL compared to type II EATL whereas gain of *MYC* at 8q24 is less frequent in type I EATL than in type II EATL (27% vs. 73%). A recent study reported aCGH results for five Korean patients with type II EATL. Gains of 9q33-q34, 6p21.21 and 19q21 and loss of 3q26.31 were observed in at least two cases. In keeping with the findings of Deleeuw et al. the frequency of 9q gain was 80% and gain of 8q24 was seen in one patient [442].

Hepatosplenic T-Cell Lymphoma

Hepatosplenic T-cell lymphoma (HSTL) is a mature cytotoxic T-cell lymphoma involving the sinusoids of the spleen, liver and bone marrow that mainly affects young adults and children and can be seen in the setting of immunosuppression. Most tumours are derived from T-cells of the γδ lineage but rare cases that express TCR αβ heterodimers are also reported [452]. In cases where conventional cytogenetics has identified an abnormal clone, the modal number is most often in the near-diploid range and a mixture of abnormal and normal metaphases is usual. Isochromosome of 7q is the most common cytogenetic abnormality in HSTL [453–457]. Since a stemline with an isolated i(7q) is often encountered together with sidelines containing additional cytogenetic abnormalities, the i(7q) is thought to be the primary cytogenetic abnormality in this condition. More recently variants resulting in gain of 7q as a result of ring chromosome

formation have been reported [458–460]. It is unclear if loss of 7p material or gain of 7q material is of greater consequence in i(7q) formation in HSTL. However, rare cases that demonstrate gain of 7q without coincident 7p loss, or gain of additional copies of 7q with disease progression favour gain of genetic material on 7q as the event most likely to confer selective advantage on the neoplastic clone [458, 461, 462]. Ferreiro et al. used high resolution array CGH to study six cases of HSTL with i(7q) and three cases with r(7) and localised the common deleted region to 7p at 7p22.1p14.1 and the common gained region to 7q22.11q31.1 [463].

In most reported HSTL cases with i(7q) the karyotypes are relatively simple with no more than three abnormalities. Trisomy 8 is the most common secondary abnormality described, followed by loss of the Y chromosome [453–455, 458, 459]. Isochromosome of 7q is not specific for HSTL and has also been reported in other mature T-cell neoplasms, T-cell and B-cell acute lymphoblastic leukaemia, acute myeloid leukaemia, myelodysplastic syndromes and Wilm tumour. The difference is that in conditions other than HSTL the i(7q) is usually present in complex karyotypes, as a secondary abnormality or seen to arise during clonal evolution with disease progression. Thus, the combination of i(7q) with trisomy 8 in a relatively simple karyotype is most characteristic of HSTL.

However, HSTL cases without i(7q) also exist, including cases with increased complexity [464]. A case series investigating the frequency of TCR translocation events and isochromosome 7q formation in T-cell lymphomas did not identify any abnormalities in four cases of HSTL [441]; although at least three of these cases were confirmed to be of the αβ type in which i(7q) is infrequent [462]. An early study had suggested that i(7q) was an adverse prognostic factor in HSTL [465] but this was not borne out in retrospective analysis of a larger case series [464]. Yabe et al. identified trisomy 8 in 8 of 24 patients (33%) and showed that the trisomy 8 patients had a significantly shorter overall and event-free survival. However, these patients were not treated on a uniform protocol [457].

Mycosis Fungoides/Sézary Syndrome

Primary cutaneous T-cell lymphomas (CTCL) represent a group of malignancies of mature T-cells that manifest primarily in the skin. The most common CTCL is mycosis fungoides (MF) which presents with skin lesions containing clonal malignant T-cells but CTCL may also present in a leukemic form with skin involvement and lymphadenopathy, Sézary syndrome (SS). SS is an aggressive form of CTCL defined by the triad of erythroderma, generalised lymphadenopathy and the presence of neoplastic T-cells in skin, lymph node and peripheral blood. Approximately 10–20% MF transforms to a large T-cell lymphoma over time.

Conventional cytogenetic studies of CTCL have been hampered by the difficulty in producing good quality metaphase spreads from malignant cutaneous lymphocytes, which may be mixed with a large number of reactive lymphocytes. In consequence, low-grade CTCL have appeared more likely to have a normal karyotype. In contrast, high-grade CTCL have been reported to have deletions of 6q and 13q and trisomy of 7q; these abnormalities have been shown to involve deletion of 6q21, amplification of 7q21 and interstitial deletion of 13q14 [466].

A small study by Thangavelu et al. of 19 patients with SS / MF identified clonal abnormalities by conventional cytogenetics in 53% of cases [467]. The commonest abnormalities were deletions of 1p22-1p36, rearrangements involving chromosomes 10 and 17 and, in two cases, a der(8)t(8;17)(p11;q11) was seen. Regions of the genome encoding T-cell receptors were not involved in abnormalities.

Espinet et al. [468] studied another 21 patients with SS using both conventional cytogenetics and cross-species colour banding and found the most common structural abnormalities to involve 1q, 2q, 6q and 8q and the most common numerical abnormalities to be monosomies of 9 and 10 and trisomy 18 with monosomy 10 in 73% of abnormal cases. Multicolor FISH applied to 9 abnormal MF/SS karyotypes also showed abnormalities of 10q to be the most common, observed in 7/9 cases [469]. Recurrent breakpoints were: 1p32-36, 6q22-25, 17p11.2-13, 10q23-26 and 19p13.3 and one patient also showed the dicentric 8;17 translocation described previously in two patients with SS by Thangavelu et al. [467]. Loss of heterozygosity was examined at a number of loci in 19 MF patients and frequent LOH confirmed at 10q23 (*PTEN*) and 17p13 (*TP53*) [470].

Array data from a number of publications is summarised in Table 42.3. The most frequently reported copy number gains are of 17q and, in two series of SS cases, gain of 8q encompassing *MYC* was observed. The most frequent recurrent losses were of 10q and 17p.

Comparing aCGH studies of 22 patients with tumour-stage MF and 20 SS patients did not identify much overlap in the recurrent chromosome aneuploides seen in the two groups [471, 472]. Five SS patients and 2 MF patients were studied using a genomic microarray containing 57 oncogenes and showed oncogene copy number gains of *RAF1* (3p25), *CTSB* (8p22), *PAK1* (11q13) and *JUNB* (19p13) in 5/7 patients. A further 23 SS and 10 MF patients were subsequently studied by immunohistochemistry and showed expression of nuclear JUNB in 21/23 SS and 5/10 MF patients [473]. Salgado et al. studied 41 MF cases by aCGH and found that gain/amplification at 8q24 (*MYC*) and deletions of 9p21 (*CDKN2A/B*, *MTAP*) and 10q26-10qter (*MGMT*, *EBF3*) were significantly associated with shortened survival. They also made the observation that patients with

Table 42.3 Array data showing recurrent copy numbers gains and losses in MF and SS

Dx (no. studied)	Gains	Losses	References
MF (41)	**7q33.3-7q35**, **17q21.1**, **8q24.21**, 9q34-9qter, 10p14	**9p21.3**, **9q31.2**, 17p13.1, 13q14.11, 6q21.3, 10p11.2, 16q23.2, 16q24.3	[474]
MF (22)	1p36.2, 1q31-1q32, **multiple regions on 7q and 7p**	5q13, 9p21, 13q14-13q31	[472]
MF (16), SS (18)	4/4q, 17q/17, 18	1p31–36, 10q26, 17p, 19	[559]
MF (3), SS (4)	17q11.2-17q12)[a]	10q23	[476]
MF (15), SS (11)	–	10q23.33–24.1, 10q24.33-10q25.1	[475]
MF-T[b] (11)	1p36, 7, 9q34, 17q24-qter, 19	2q36-qter, 9p21, 17p	[560]
SS (20)	2p11.2, 4p16.1, 7p21.1, **8q24.1-q24.3**, **17q21.31-q23**	3q26.33, 5q14.3, 7p14.1, 9p13.1-9p2, **multiple regions on 10**, 16p11.2, **17p13-17p12**	[471]
SS (28)	**17p11.2-17q25.3**, **8/8q**, 10p15.3-10p12.2	**10p12.1-10q16.3**, **17p13.2-17p11.2**, 9q13-q21.33	[561]
SS (6)	22q11.2-22q13.3	1p36-1p22, 6q24, 15q11.2	[562]

Chromosome regions in bold represent the commonest aberrations observed in the study
[a]Encompassing *ERBB2 (HER2)*
[b]MF-T: mycosis fungoides undergoing large cell transformation

genomically unstable tumours (those with >5 copy number changes) had significantly worse outcomes [474].

Deletions of 10q23.33-10q24.1 and 10q24.33-10q15.1 were confirmed in 43% MF/SS cases by microsatellite loss of heterozygosity studies [475] and duplication of 17q11.2-17q12 has been demonstrated by aCGH covering the region of the genome that contains the *ERBB2 (HER2)* and STAT family genes in both MF and SS, suggesting it may be a primary karyotypic event common to both disorders [476]. FISH for the *ERBB2 (HER2)* gene locus at 17q11.2-q12 has shown gain of copy number in 7/9 SS cases [477].

In 2005, Karenko et al. [478] reported clonal deletions or translocations affecting chromosome 12 with a breakpoint in 12q21 or 12q22 in 5/7 SS patients and a sixth patient with a clonal monosomy of chromosome 12. A reciprocal t(12;18)(q21;q21.2) in one patient was found to disrupt the *NAV3* gene. Locus specific FISH proceeded to show *NAV3* deletions in 50% early stage MF patients and 85% advanced MF or SS cases. However, subsequent FISH and microarray studies of advanced MF and SS patients failed to confirm the frequency of *NAV3* deletion or disruption [474, 479]. A 2011 FISH study in cutaneous T-cell lymphoma reported that translocations involving the T-cell receptor loci *TRA/TRD* (14q11.2), *TRB* (7q34) and *TRG* (7p14) were uncommon in these conditions. No *TCR* gene rearrangements were identified in the 13 SS and 6 MF cases tested [480].

Ungewickell et al. identified a group of SS cases with mutations or copy number gain of the *TNFRSF1B* at 1p36.22 resulting in activation of non-canonical NFKB signalling. *TNFRSF1B* alterations were enriched in SS cases compared to MF cases. Although they were associated with large cell transformation and reduced survival, they did not predict inferior outcomes in multivariate analysis [481]. Other recurrent abnormalities in this cohort were largely exclusive of *TNFRSF1B* alternations. They included deletions of the *NFKB2* and *TRAF3* genes (at 10q24 and 14q32, respectively) as well as *CTLA4-CD28* gene fusions and structural rearrangements involving the *CD274* (*PDL1*) at 9p24.1, the *TNFRSF10A* at 8p21 and the *TP63* gene at 3q28. Transient response to ipilimumab, an anti-CTLA4 antibody has been reported in a case of SS containing a *CTLA4-CD28* fusion [482]. *CTCLA4-CD28* fusions are also described in AITL and PTCL-NOS, whilst *TP63* rearrangements are also a feature of other lymphomas, including DLCBL, PTCL NOS and ALK-negative ALCL.

Peripheral T-Cell Lymphomas (Not Otherwise Specified) (PTCL NOS)

The genetics of PTCL NOS has not been extensively studied. A review of the cytogenetic findings of 58 cases showed the most frequent gain to be at 7q with minimum overlapping region at 7q22q31 (in 33% cases) and most frequent losses occurred at 6q22q24 and 10p13pter with each detected in 26% cases. Five cases had translocations involving band 14q11.2 at the region of the *TRA/TRD* genes and three contained rearrangements involving 11q23 [483].

A SNP array study of 47 cases identified recurrent gains of chromosome regions 1q32-43, 2p15-16, 7, 8q24, 11q14-25, 17q11-21 and 21q11-21 (in five or more cases each) and recurrent losses at 1p35-36, 5q33, 6p22, 6q16, 6q21-22, 8p21-23, 9p21, 10p11-12, 10q11-22, 10q25-26, 13q14, 15q24, 16q22, 16q24, 17p11, 17p13 and Xp22 (four or more cases each). Gain of the *REL* locus on 2p was detected by FISH in another 3/18 further cases studied and 5/27 PTCL NOS showed nuclear expression of REL by immunohistochemistry [484]. Another study using aCGH of 51 PTCL NOS identified DNA gains and losses in 29 cases (57%) with high copy number gain at 14q32.2 and homozygous loss at 9p21.3. Gains of 7p and 7q and loss of 9p21.3 showed a significant association with poor prognosis [440].

Zettl et al. used chromosome comparative genomic hybridisation to identify recurrent chromosomal losses at

13q21, 6q21, 9p21 to 9pter, 10q23-24, 12q21-q22 and 5q21 in 25% or more of PTCL NOS cases. Recurrent gains of chromosome 7q22-qter were observed in 31% cases with high-level amplification of 12p13 observed in three cases [485]. The cyclin dependent kinase 6 gene (*CDK6*) has been identified as a potential target for 7q22 amplification. Amplification of *CDK6* was demonstrated by interphase FISH on paraffin-embedded tissue sections in 5/23 (23%) PTCL NOS cases [486]. Yoshida et al. identified a minimal common deleted region in PTCL NOS at 6q24 containing just two genes: *STX11* and *UTRN* [487]. Expression of STX11 was reduced in PTCL cases and loss of function mutations were identified in one PTCL patient and T-cell lymphoma cell line. Interestingly, *STX11* deletion or mutation was associated with haemophagocytosis and germline mutations in *STX11* result in the autosomal recessive condition familial haemophagocytic lymphohistiocytosis type 4. Collectively, these studies suggest a role for *STX11* as a haploinsufficient tumour suppressor gene in PTCL NOS.

A SNP array study of 33 PTCL NOS and 40 angioimmunoblastic T-cell lymphomas (AITL) detected recurrent copy gain common to both conditions on chromosomes 8, 9 and 19 with common LOH detected on chromosome 2. Poor prognosis was linked to overexpression of *CARMA1* at 7p22 and of *MYCBP2* at 13q22, both being located within regions of frequent copy gain [488]. Truncated forms of *IKZF2* at a frequently deleted region at 2q34 were also identified.

Translocations involving *IRF4/DUSP22* locus on 6p25 have been observed in PTCL NOS cases. A study of 72 PTCL NOS cases revealed two with the t(6;14)(p25;q11.2) fusing *IRF4* and T-cell receptor alpha (*TRA*), both of which presented in the bone marrow and rapidly developed skin lesions, and also another case with a translocation involving *IRF4* but not *TRA* [489]. Occasional other cases of the t(6;14) have also been reported [490, 491].

The t(14;19)(q32;q13) has been observed in a variety of B-cell lymphomas; there have also been occasional reports of a t(14;19)(q11.2;q13) involving the *TRA/TRD* gene locus and resulting in nuclear BCL3 protein expression [450, 492]. Another three cases of t(14;19)(q11.2;q13) had the 19q13 breakpoint localised to within or downstream of the *PVRL2* gene; two of these cases showed high mRNA levels of both *BCL3* and *PVRL2* [493]. Shin et al. reported a Lennert's type (lymphoepitheloid) PTCL-NOS with a t(14;19)(q11.2;q13.3) involving *TRA/TRD* where the breakpoint on chromosome 19 was located distal to both genes, implicating a putative oncogene other than *BCL*3 or *PVRL2* [494]. Other cytogenetic abnormalities that are well-recognised in various subtypes of T-cell lymphomas such as translocations involving T-cell receptor genes and isochromosome of 7q are reportedly uncommon in PTCL NOS [441].

Angioimmunoblastic T-Cell Lymphoma

Angioimmunoblastic T-cell lymphoma (AITL) is a well-defined histopathological entity but there are few comprehensive studies of the genetics of this disorder. AITL cases express T-cell follicular helper (TFH) associated antigens such as CD274 (PDL1), CD10, BCL6, CXCL13, ICOS, SAP and CCR5. It has been recognised that two other peripheral T-cell cell lymphoma types, namely follicular T-cell lymphoma (FTCL) and nodal PTCL with TFH phenotype, also share a TFH phenotype and fall under the AITL listing in the 2016 revision of the WHO Classification [187]. There are two classes of gene mutations common to this group: firstly, mutations in epigenetic modifiers such as *TET2*, *IDH2* and *DNMT3A* that also have an association with myeloid malignancies, and secondly, mutations in the *RHOA* and *CD28* genes that are specific to T-cell disorders. There are also two recurrent chromosomal rearrangements which are associated with but not exclusive to lymphomas in this group: the t(5;9)(q33;q22) and a cryptic 2q33 rearrangement resulting in formation of a *CTLA4-CD28* gene fusion.

The t(5;9)(q33;q22) was identified in PTCL NOS and the breakpoints shown to involve the *ITK* and *SYK* genes on chromosomes 5 and 9, respectively. The translocation resulted in overexpression of the *SYK* gene under the control of the *ITK* promoter. The translocation was demonstrated in 5/30 (17%) PTCL cases, three of which were FTCL, but was not found in cases of AITL or anaplastic large cell lymphoma [495]. Overexpression of the *SYK* tyrosine kinase has been demonstrated by immunohistochemistry in 94% of 141 PTCL, in the absence of the t(5;9), whereas normal T-cells were negative [496]. Although there is at least one report of the t(5;9) occurring in a case of AITL [497], other studies confirmed association of the t(5;9) with FTCL [498] and the rarity of the t(5;9) in AITL [499].

CTLA4-CD28 fusions have been described in a variety of T-cell lymphomas including Sézary syndrome [481, 482]. Yoo et al. reported *CTLA4-CD28* fusions in 26/45 (58%) AITL, 9/39 (23%) PTCL-NOS and 9/31 (29%) extranodal NK/T-cell lymphomas [500]. However, frequent occurrence of the *CTLA4-CD28* fusion in these disorders has not been confirmed by other investigators [501].

Trisomy 3, trisomy 5 and an extra copy of X have been identified as recurrent abnormalities in most AITL cases [466] and trisomies of 3 and 5 were confirmed as typical abnormalities in another 20 AITL cases [502]. However, an aCGH study of 39 AITL cases revealed chromosomal imbalances in 72% with recurrent gains of 22q, 19 and 1p11-11q14, and recurrent losses of 13q and found +3 and +5 in only a small number of cases [503]. Interestingly, FISH studies have shown the trisomies to occur frequently in separate cells, suggesting the presence of multiple, possibly unrelated clones. Schlegelberger et al. [504] suggested that an addi-

tional X was associated with a shorter survival but trisomy 3 did not appear to impact on survival. FISH using break-apart probes for the *TRA/TRD* and *TRB* genes did not show any evidence of *TRA/TRD* or *TRB* translocation events in 24 cases studied [505]. Tissue microarray FISH identified 9p24 gains in 3/16 (19%) AITLs studied. Increased copy number correlated with increased proportions of tumour cells expressing phosphorylated JAK2 and phosphorylated STAT3 [338].

Anaplastic Large Cell Lymphoma (ALK Positive)

Anaplastic large cell lymphoma (ALCL) is a mature T-cell/null cell lymphoma, which may present with systemic or localised cutaneous growth. Systemic ALCL is characterised by deregulated expression of anaplastic lymphoma kinase (ALK) fusion proteins. Shortly after its initial morphological description, ALCL (ALK positive) was found to be characterised by a 2;5 translocation [506]. The t(2;5)(p23;q35) forms a fusion gene *NPM-ALK* on the derivative chromosome 5. *NPM* on 5q35 encodes an RNA-binding protein that shuttles ribosomal ribonucleoproteins between the nucleus and the cytoplasm. *ALK* on 2p23 encodes a receptor tyrosine kinase, a member of the insulin receptor subfamily of RTKs. The fusion gene formed by the t(2;5) causes the strong *NPM* promoter to drive transcription of the portion of *ALK* encoding its kinase domain, leading to its inappropriate expression in lymphoma cells. The resulting fusion protein can be identified within both the cytoplasmic and the nuclear/nucleolar compartments by immunostaining.

Ten to twenty per cent of *ALK*-positive lymphomas show *ALK* expression only in the cytoplasm. These cases usually represent *ALK* fusions with partners other than *NPM*. A number of variant translocations with cloned partner genes have now been described (see Table 42.4) and there are still further *ALK* rearrangements reported with the partner genes yet to be determined [506–514]. All of the *ALK* chromosomal aberrations lead to the expression and constitutive activation of ALK and the clinico-pathological features of cases with variant *ALK* fusions appear indistinguishable from *NPM-ALK*-positive cases.

Additional chromosomal abnormalities have been observed in most cases. Three paediatric cases of ALCL with t(2;5) showed complex aberrations ranging from 3 to 11 with both numerical and structural abnormalities [515]. Rare cases with a *MYC* translocation in addition to the t(2;5) have been described and appear to be associated with an aggressive clinical course [516, 517]. Array CGH was performed on biopsy material from 15 children with ALK-positive ALCL. Regions of genomic gain (range 1–38 per patient) were more common than regions of loss (range 0–3 per patient). The total number of aberrations ranged from 2 to 40. The highest number of gains was on chromosome 11 (in 93% patients) and imbalances were demonstrated at a number of regions on 11p and 11q. Gain of 7q36.3 was observed in 67%. Regions of loss were not associated with known tumour suppressor genes. A lower overall 3-year survival was associated with higher numbers of genomic imbalances, gains of the 11q12.2 region containing the *DDB1* gene and gains of 17q25.3 containing the *BIRC5* gene [518]. A study by Salaverria et al. of 43 ALK-positive and 31 ALK-negative ALCL using a combination of chromosome CGH, FISH and quantitative PCR identified chromosomal imbalances in 25/43 (58%) and 20/31 (65%), respectively. Both groups exhibited gain of chromosome 7 and loss of 13q but ALK-positive ALCL were more likely to show gains of 17p and 17q24-qter and losses of 4q13-21 and 11q14 [519]. No specific chromosomal aberrations were correlated with survival.

Table 42.4 Known *ALK* partner genes in ALCL

Fusion Gene	Chromosomal abnormality	Staining pattern of ALK protein	% of cases (%)
NPM-ALK	t(2;5)(p23;q35)	Nuclear and cytoplasmic	84
TPM3-ALK	t(1;2)(q25;p23)	Cytoplasmic	13
ATIC-ALK	inv(2)(p23q35)	Cytoplasmic	1
TPM4-ALK	t(2;19)(p23;p13.1)	Cytoplasmic	<1
TFG-ALK[a]	t(2;3)(p23;q21)	Cytoplasmic	<1
MSN-ALK	t(X;2)(q12;p23)	Membrane	<1
CLTC-ALK	t(2;17)(p23;q23)	Cytoplasmic (granular)	<1
MYH9-ALK	t(2;22)(p23;q11.2)	Cytoplasmic	<1
ALO17-ALK	t(2;17)(p23;q25)	Cytoplasmic	<1
TRAF1-ALK	t(2;9)(p23;q33)	Cytoplasmic	<1

Data derived from references [506–514]
[a]There are three different variants of this translocation: $TFG\text{-}ALK_s$ (short), $TFG\text{-}ALK_l$ (long) and $TFG\text{-}ALK_{xl}$ (extra long)

ALK-positive lymphomas may be identified by conventional cytogenetics, FISH, PCR and immunohistochemical staining using anti-ALK antibodies. FISH probes that span the *ALK* locus are available and identify *ALK* translocations in ALK-positive lymphomas irrespective of the translocation partners. FISH works reliably on both metaphase chromosomes and interphase cells.

Anaplastic Large Cell Lymphoma (ALK Negative)

In 2011, Feldman et al. used massively parallel sequencing techniques to identify a recurrent t(6;7)(p25.3;q32.3) translocation in ALK-negative ALCL associated with

downregulation of DUSP22 at 6p25.3 and upregulation of MIR29 at 7q32.3 [520]. King et al. reported that *DUSP22* rearranged cases of ALCL were characterised morphologically by a sheet-like growth pattern, doughnut cells and the absence of pleomorphic cells [521]. In 2012, Vasmatzis and colleagues described rearrangements in the *TP63* gene at 3q28 in 5.8% of PTCLs and 1% of DLBCL cases [522]. The most common abnormality was a paracentric inversion of chromosome, inv.(3)(q26q28), involving *TBL1XR1* gene at 3q26 and *TP63* at 3q28. The inversion generated a fusion protein with loss of the N-terminal transactivation domain of TP63 and a dominant-negative effect on other members of the TP53 family.

No gene rearrangements are specific to ALK-negative ALCL. However, larger screening studies using break-apart probes indicated that *DUSP22* and *TP63* gene rearrangements were present in approximately 30% and 8% cases, respectively. Parilla Casteller et al. showed that *DUSP22* rearranged cases had favourable clinical outcomes, equivalent to ALK-positive ALCL. The prognosis in *TP63* rearranged cases was poor, whilst cases lacking both a DUSP22 and a TP63 rearrangement had an intermediate prognosis [523].

In terms of copy number changes, the study by Salaverria et al. of 43 ALK-positive and 31 ALK-negative ALCL identified chromosomal imbalances in 20/31 (65%) ALK-negative ACLC [519]. Whilst both ALK-positive and ALK-negative groups exhibited gain of chromosome 7 and losses of 6q and 13q, gains of 1q and 6p21 were more frequent in ALK-negative ALCL. ALK-negative ALCL was also reported to contain trisomy 2 leading to an extra copy of the *ALK* gene but without showing any *ALK* gene rearrangements [524].

Hodgkin Lymphoma

The diagnosis of Hodgkin lymphoma (HL) is a histopathologic one and depends on demonstration of characteristic neoplastic Hodgkin Reed-Sternberg (HRS) cells or LP (lymphocyte predominant) cells in Classical Hodgkin lymphoma (CHL) and Nodular lymphocyte predominant Hodgkin lymphoma (NLPHL), respectively.

Study of HL using conventional cytogenetic techniques has been limited with less than 250 aberrant cases published to date, in part reflecting the challenges of standard techniques in this disease. Tumours often have a low mitotic index in vitro and as the neoplastic cells often make up only 0.1–10% of the tumour, large numbers of metaphases may have to be examined to overcome the problem of normal cell contamination. Furthermore optimal chromosome morphology is difficult to achieve and the extreme complexity of the abnormalities identified has precluded complete karyotyping in some instances. More recently application of molecular karyotyping to samples enriched for the neoplastic clone by way of FICTION (fluorescence-immunophenotyping and interphase cytogenetics as a tool for investigation of neoplasm), FACS, and laser microdissection with or without DNA amplification has provided important insights into the genetic changes driving these malignancies.

Classical Hodgkin Lymphoma

Classical Hodgkin lymphoma is defined by the presence of HRS and is subdivided histologically into nodular sclerosis, mixed cellularity, lymphocyte-rich and lymphocyte-depleted subtypes. To date no specific non-random, recurrent cytogenetic abnormality has been identified that correlates with any subtype, precluding a role for conventional karyotyping in disease sub classification. Conventional karyotyping generally has reported success rates of 57% [525] to 82% [526] and abnormality rates between 11% [527] and 59% [525]. In most published series abnormal karyotypes are complex with a modal number is in the triploid to tetraploid range and include both numerical and structural abnormalities. Gains of chromosomes 2, 5, 9, 11, and 12, losses of 10, 13, 16, 21 and Y and structural abnormalities involving 1p, 1q, 4q, 6q, and 7q are over-represented in most case series [525–532].

Development of the FICTION technique allowed FISH methods to be applied to non-dividing CD30+ cells within tumours [533] and analysis showed that these cells contained three or more copies of at least two of chromosomes 1, 8, 12 and 17 in most of the 30 cases studied, [533] suggesting that the CD30-positive cells harbour the near-triploid and tetraploid abnormalities observed in those cases where conventional cytogenetic studies have been successful. In 1999, Ohshima et al. [534] applied chromosome CGH to CD30+ flow sorted cells after DOP-PCR DNA amplification in nine cases of CHL and identified recurrent gain of 1p13, 12q24, and 7q35-36 and recurrent loss of 16p11-21. Joos et al. [535] performed chromosome CGH on microdissected CD30+ cells from 12 patients and found recurrent gain of 2p, 9p and 12q as well as amplification of 4p16, 4q23-24 and 9p23-24. The region of gain on 9p spanned the *JAK2* locus and *JAK2* amplification was confirmed in some tumours in this study by FISH [535]. Meier et al. reported 9p24 gain by FISH in 33% of CHL [338]. The importance of JAK-STAT signalling in CHL pathophysiology was confirmed by identification of *SOCS1* loss of function mutations resulting in deregulated STAT activity in Hodgkin lymphoma [341] after they were initially described in the related condition primary mediastinal B-cell lymphoma (PMBL) [340]. Subsequently, Green et al. [342] identified *CD274* (*PDL1*) and *PDCD1LG2* (*PDL2*) as key targets of 9p24 amplification in HRS cells

from nodular sclerosis CHL and in primary mediastinal B-cell lymphomas (PMBL) using combined aCGH data and gene expression profiling. *JAK2* was co-amplified and contributed to PD-1 ligand induction via a forward feedback loop. Since PDL1 and PDL2 are T-cell inhibitory ligands the authors proposed that deregulation may promote tumour immune escape in nodular sclerosis HD and PMBL [342]. Van Roosbroeck et al. screened 200 cases of CHL by interphase FISH for 9q24.1 aberrations and, as well as finding *CD274/PDCD1LG2* rearrangements in four (2%), also showed high-level amplification of 9p24.1 in 40 (25%) including four cases with selective *CD274/PDCD1LG2* amplification [536].

FICTION studies implicated *REL* as the candidate oncogene at 2p13 [537]. In 2001, Kupper et al. confirmed gain of the 12q14 locus, encompassing *MDM2* by interphase FISH on CD30+ cells [538]. The same group proceeded to analyse a larger cohort by CGH on HRS cells and showed gains of 2p15-p16 (the only region involved in more than 50% of cases) and 12q24 to be the most frequent recurrent gains followed by gain of 17p, 9p, 16p, 17q and 20q together with loss of 13q. The *REL* gene was included in the 2p15 amplicon and 2p gain was particularly enriched in the nodular sclerosis CHL subtype (88% of cases) [539]. The CGH findings of Joos et al. [535] were largely confirmed in a similar independent study which reported frequent gain of 17q, 2p, 12q, 17p, 22q, 9p, 14q, and 16p and loss of 13q, 6q, 11q and 4q using a comparable experimental approach [540]. Given the scarcity of HRS cells, rather than using FICTION, Salipante et al. isolated HRS cells by flow cytometric cell sorting before performing whole genome sequencing to detect genomic aberrations. Recurrent copy number aberrations involving *REL*, the PD-1 pathway and *TNFAIP3* were observed, and loss of the chromosome 1 region containing tumour suppressor *TNFRSF14* was observed in 8/19 (42%) cases [541].

A major limitation of CGH is the inability to detect balanced reciprocal translocations; however, their presence in CHL has been investigated using FISH-based translocations assays. Using a BCL3 FISH break-apart probe, the *BCL3* gene (also known as *RELB*) at 19q13.2 was shown to be either involved in translocations in CHL, including one t(14;19)(q32;q13.2), or to demonstrate gains greater than ploidy level in patients with CHL [542], in keeping with the *BCL3* rearrangements or copy number changes observed in CD30+ HL cell lines [492]. However *BCL3* rearrangements are also described in other lymphomas and are not specific to CHL. As 14q32 rearrangements had a reported frequency of 10–20% by conventional cytogenetics [526, 532, 543], a later study of 232 patients with CHL set out to determine the frequency of translocations involving the *IG* loci and to identify the translocation partners using FISH and FICTION techniques [544]. The investigators identified *IG* rearrangements in 20% of cases with involvement *IGH* being much more common than involvement of the kappa and lambda light chain loci. This result places translocations involving immunoglobulin loci amongst the three most frequent cytogenetic abnormalities in CHL, after 2p13-16 and 9p24 gain. Where translocation partners could be identified they included *BCL6*, *REL*, *BCL3* and *MYC*, demonstrating that *BCL6* translocations were not confined to or diagnostic for NLPHL HL. No translocations involving *CCND1*, *BCL2*, *MALT1* or *PAX5* were detected. In most cases, immunoglobulin translocation events appeared to be the result of erroneous class switch recombination events consistent with a germinal centre B-cells as the putative cell of origin in CHL in most cases. A t(4;9)(q21;p24) translocation has been observed in a small number of CHL cases, causing a *SEC31A-JAK2* fusion and making CHL cases with the translocation potentially amenable to JAK inhibitor therapy [545]. There have been rare cases of HL associated with follicular lymphoma. The t(14;18) is rarely seen in HL but Yoshida et al. demonstrated its presence via chromogenic in situ hybridisation and FISH in three cases of HL associated with FL [546].

HRS cells in CHL show constitutive activation of NFKB and genetic abnormalities of NFKB regulators have been identified: deletion of the *TRAF3* gene was seen using interphase FISH analysis in 3/20 primary CHL samples and gain of the key signalling component of the alternative NFKB pathway *MAP3K14* (17q21) was observed in 5/16 cases [547].

Hartmann et al. [548] studied CHL by aCGH. The authors took DNA extracted from pooled microdissected HRS cells from twelve highly unusual tumours comprised of over 70% HRS cells (in order to avoid introduction of biases inherent in DNA amplification) and hybridised them to 105K oligonucleotide arrays. They were able to obtain data of sufficient quality for subsequent analysis in ten cases. Copy number changes were extensive, gains were more frequent than losses, consistent with data from metaphase CGH studies, and many imbalances detected by CGH were confirmed. In addition novel regions of chromosomal imbalance were identified: 2p12-16, 5q15-223, 6p22, 8q13, 8q24, 9p21-24, 12q13-14, 17q12, 19p13, 19q13 and 20q11 were gained and Xp21, 6q23-24 and 13q22 were lost in at least 50% of cases, respectively. NFKB signalling is known to be constitutively active in CHL and, strikingly, again areas of gain and loss often involved NFKB family members including gain of *REL* at 2p, *BCL3* at 19q13, *RELA* at 11q23, *NFKB2* at 10q24, *CARD9* and *TRAF2* at 9q34, *JUNB* at 19p13 and deletion of *TRAFAIP3* at 6q23-24, all of which are predicted to perturb NFKB signalling. Intriguingly, lesions also involved noncoding microRNA loci but the functional significance of these changes for CHL biology remains to be determined.

Steidl et al. [549] performed aCGH analysis after whole genome amplification on microdissected HRS cells from 53

cases of CHL, including some patients who experienced primary treatment failure. The most frequent imbalances were gains of 2p15-16.1, 9p21.1, 9p24.1-24.3, 16p11.2-13.3, 17q21.21-32, 19q13.11-13.43 and 20q13.11-13.12 and losses of 6q23.2, 11q22.3 and 13q14.3-21.1 demonstrating considerable overlap with and validating the findings of Hartmann et al. [548]. Further novel findings from this study again centred on the NFKB signalling pathway. Gains on 20q involved the CD40 locus; canonical and non-canonical NFKB signalling are downstream of this TNF receptor family member that is frequently overexpressed in CHL. In addition there was an increase in *IKKB* copy number at 8p11.21, which may facilitate nuclear localisation of NFKB by targeting IKBA and IKBE for proteosomal degradation. This study refined the recurrent region of gain on 17q to a 3.9 MB region containing *MAP3K14*, a gene that encodes the NFKB inducing kinase (*NIK*) and plays a critical role in non-canonical NFKB signalling. Perhaps most notably, gain of 16p11.2-13.3 was associated with primary treatment failure in CHL in this relatively large cohort. One candidate gene mapping to this retained region is the multi-drug resistance gene *ABCC1*. It is possible ABCC1 does indeed mediate the inferior clinical outcomes seen in the cohort with 16p gain as the authors went on to demonstrate that knockdown of ABCC1 using siRNA particularly in CHL cell lines with 16p copy number gain restored anthracycline induced cytotoxicity in vitro [549].

Nodular Lymphocyte Predominant Hodgkin Lymphoma

NLPHL makes up just 5% of cases of HL, and as a consequence of its relative rarity has been much less extensively studied that CHL. Much as these tumours are set apart from CHL on the basis of their distinct clinico-pathological phenotype, it is also becoming apparent that these phenotypic differences are underpinned by discrete changes at the genetic level.

Conventional cytogenetic analysis in NLPHL has identified a clonal population in less than 25 cases, most of which were hyperdiploid with complex karyotypes [296, 525, 532, 550–553]. Franke et al. [298] performed metaphase CGH after DOP-PCR of DNA obtained from laser-microdissected CD20+ LP cells in 19 patients. The mean number of copy number changes was 10.8 reflecting at a molecular level the complexity observed in metaphase preparations. Chromosomal regions most frequently gained were 1p, 1q, 2q, 3p, 3q, 4q, 5q, 6p, 6q, 8q, 11q, 12q and X and the regions most frequently lost were 17 and 17q. This data set was not enriched for loci encoding genes involved in NFKB signalling in contrast to the pattern of recurrent chromosomal imbalances present in CHL. A 3q27 breakpoint was observed in two cases in this cohort where conventional cytogenetics was informative and FISH confirmed *BCL6* rearrangement. A follow-up study reported *BCL6* rearrangement in 11/23 (48%) NLPHL cases but in none of the 40 CHL patients tested [554]. Furthermore, interphase FISH/FICTION studies by an independent group to determine the frequency of immunoglobulin rearrangements in NLPHL identified aberrant signals in 5/24 tumours studies and all five cases were shown to have a reciprocal 3;14 translocation giving a frequency of 21% [555]. As *BCL6* rearrangement is common in DLBCL and less common but reported to occur CHL by other investigators [544], this abnormality is enriched in NLPHL patients but cannot be considered specific to this HL subtype. *BCL6* rearrangement in NLPHL has been shown to occur in CD20+ cells, to correlate with strong protein expression of BCL6, to involve both immunoglobulin and non-immunoglobulin translocation partners and can manifest as simple balanced reciprocal translocations or more complex rearrangements [554, 556]. Using FISH, Bakhirev et al. identified *BCL6* rearrangements in 5 of 11 (45%) cases of NLPHL but also saw *BCL6* gene amplification with large clusters of BCL6 signals without an increase in copies of chromosome 3 in 3 of 11 (27%) cases and extra copies of *BCL6* in the setting of multiple copies of chromosome 3 in 1 of 11 (9%) cases. Thus, *BCL6* aberrations were shown to be present in 9 of 11 (82%) cases [557].

In 2007, Stamatoullas et al. published the largest and most comprehensively karyotyped series of NLPHL patients [296]. This group was able to obtain analysable metaphases from all 13 patients with a confirmed diagnosis of NLPHL and identified clonal abnormalities in 12/13. All had complex karyotypes with complex defined as greater than three abnormalities and in most cases clonal abnormal metaphases were outnumbered by spreads with a normal karyotype. Striking, 11/12 patients had a model number in the near-diploid range with the exception being a patient with a tetraploid karyotype, suggesting near-triploidy is much more uncommon in this disorder than it is in CHL. Isochromosome of 1q ($n = 7$) or unbalanced translocation resulting in gain of 1q ($n = 3$) was the most common recurrent abnormality, followed by a high frequency of 3q27 and 14q32 breakpoints and frequent involvement of *BCL6* and *IGH* was confirmed by FISH, validating the findings of Wlodarska et al. [554] and Renne et al. [555]. However, this study failed to identify many of the copy number changes detected by CGH [298]. This study proposes near-diploid karyotypes, gain of 1q and *BCL6* translocations as some of the more distinctive cytogenetic features of NLPHL, although some of these findings are yet to be confirmed by other investigators and aCGH studies in particular are lacking.

Thus far, cytogenetic studies in HL have proved invaluable research tools for understanding tumour cell biology in CHL and NLPHL. Whilst showing some recent promise, the

abnormalities uncovered thus far lack specificity as biomarkers and have not demonstrated prognostic impact in independently validated studies. Therefore, to date cytogenetic studies have not been incorporated into routine clinical decision-making algorithms in these conditions.

Summary

Conventional cytogenetic analysis and FISH are now an integral part of the diagnosis of some but not all lymphoid malignancies. These tests are critical for the diagnosis of patients with mantle cell lymphoma and Burkitt lymphoma and for the identification of patients with high-grade B-cell lymphoma and "double-hit" or "triple-hit" *MYC*, *BCL2* and *BLC6* translocations. Both conventional cytogenetics and FISH panels are used to determine prognosis in chronic lymphocytic leukaemia and the finding of a t(11;18) in a MALT lymphoma predicts failure of *H. pylori* eradication therapy. However, for most lymphoma subtypes, although there are frequent cytogenetic abnormalities detected by a variety of methods, such testing does not materially add to the determination of diagnosis or outcome and so is not part of the routine testing algorithm for most lymphomas.

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Diagnosis and Treatment of Hodgkin Lymphoma

43

Peter H. Wiernik

Introduction

The successful treatment of Hodgkin lymphoma is one of the most impressive achievements of modern medicine. Today more than 90% of patients without fever, weight loss, or night sweats (B symptoms) are cured with optimal treatment, and 60–70% of those with B symptoms are cured as well. Fortunately, most patients do not have B symptoms at presentation. These cure rates have not been achieved without problems. Long-term complications of cure have interrupted the well-being of a sizeable fraction of survivors, and relapses—even late relapses—are still a problem. Current clinical research is focused on optimal noninvasive staging of disease, reducing the short- and long-term complications of treatment, and early diagnosis of serious long-term complications such as cardiac disease and second malignancies. New drugs with major activity against this lymphoma have recently been identified and their precise role in initial and late treatment is currently under investigation.

Etiology and Early Pathogenesis

Classical Hodgkin lymphoma is characterized by aberrant cytokine production which contributes to proliferation of Reed–Sternberg cells and to the maintenance of a favorable microenvironment for those cells. Especially important in this regard is overexpression of components of the TNF receptor family (CD30 and CD40) by Reed–Sternberg cells [1].

Evidence suggests that approximately 30–50% of patients with Hodgkin lymphoma, a B-cell neoplasm, harbor the Barr–Epstein virus (EBV) [2] and that the virus plays a pathogenetic role in those patients. The proportion of patients with EBV is higher in nonindustrialized countries [3] and in patients with advanced-stage disease [3]. In a Japanese study patients with CD20+ background cells in a lymph node biopsy were more likely to have EBV+ Reed–Sternberg cells than if background cells were CD20- [4]. Curiously, CD20+ background cells have a favorable effect on prognosis of patients with Hodgkin lymphoma, while EBV+ Reed–Sternberg cells have a negative effect in most studies [4, 5]. Furthermore, detection of EBV DNA in pretreatment whole blood [6] or plasma [7] is a significant negative prognostic factor. Infectious mononucleosis is a risk factor for EBV-related Hodgkin lymphoma in patients with HLA-A*01, but not in those with HLA-A*02 alleles [8]. Patients with HLA-A*01:01 or B*37:01 alleles are at increased risk for EBV+ classical Hodgkin lymphoma and those with DRB1*15:01 or DB1*01:01 are at decreased risk [9]. Patients with HLA allele E*01:01 may be protected to some degree from EBV infection as well [10]. The genetic variant, rs6457715, located at 6p21.3 near the HLA-DPB1 gene is present significantly more frequently in EBV+ Hodgkin's lymphoma than in EBV- cases, which suggests that this site may be a susceptibility locus for classical Hodgkin lymphoma [11]. HLA class I-restricted EBV-specific cytotoxic T-cell responses and other early events in the immune response to EBV infection in infectious mononucleosis play critical roles in the early pathogenesis of EBV-related Hodgkin's lymphoma. EBV-encoded small RNAs found in EBV-infected cells suppress p21cip1/waf1 transcription in Hodgkin cell lines KMH2 and L428 [3] and increase resistance to drugs known to cause apoptosis by elevating p21cip1/waf1 levels. In patients, EBV+ biopsies with suppressed p21cip1/waf1 levels were associated with an impaired disease-free survival compared with patients who were EBV-negative [12]. However, in contrast with other studies, Benharroch et al. [13] found that the presence of EBV latent membrane protein-1 in Reed–Sternberg cells did not correlate with inhibition of apoptosis in those cells. A recent authoritative review discusses the role of EBV in the pathogenesis of Hodgkin lymphoma in more detail [14].

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The JC human polyomavirus (JCV) was detected in peripheral blood lymphocytes in 42 of 74 patients with Hodgkin lymphoma in one study [15]. In that study JCV positivity was associated with chromosomal instability and a poor prognosis. However, these findings have not been confirmed [16].

The malignant cell of Hodgkin lymphoma is the Reed–Sternberg binucleate cell [17]. Salipante et al. [18, 19] described a reciprocal translocation between chromosomes 2 and 3 which disrupts a novel gene at 3p21.31, resulting in its loss of expression. Loss of this gene expression leads to an increase of binucleate cells and may be responsible for the binucleate nature of the Reed–Sternberg cell.

Reed–Sternberg cells often express insulin-like growth factor-1 receptor (IGF-1R), and patients whose cells express IGF-1R have a better prognosis [20, 21]. Reed–Sternberg cells also often express matrix metalloproteinase-9 (MMP9), especially those positive for EBV. MMP9+ patients had impaired overall survival in one study [22] but not in another [23]. Both studies are from Brazil. Reed–Sternberg cells secrete lymphotoxin-α, which facilitates recruitment of T-cells into lymph nodes of patients with classical Hodgkin lymphoma [24].

The microenvironment surrounding Reed–Sternberg cells includes immune cells, fibroblasts, stromal cells, and endothelial cells, and this microenvironment is a functional component of the lymphoma. The specific characteristics of this microenvironment have important implications for pathogenesis and prognosis. There is cross talk between Reed–Sternberg cells and the microenvironment surrounding them [25] which inhibits cytotoxic effectiveness and induces immunosuppressive T lymphocytes, predominantly CD4+ T-helper cells [26] and monocytes [25]. Reed–Sternberg cells secrete numerous chemokines to control their microenvironment. Reed–Sternberg cells themselves occasionally express T-cell antigens. When this is the case, prognosis is impaired compared with patients whose Reed–Sternberg cells do not express T-cell antigens [27]. Certain environmental T-cells provide survival signals for Reed–Sternberg cells and others rescue Reed–Sternberg cells from NK cell attack [28]. On the other hand, Reed–Sternberg cells may induce differentiation of naïve T-cells toward T-reg cells and cytotoxic T-lymphocytes, and studies have shown that increased numbers of cytotoxic T-lymphocytes compared with T-reg lymphocytes is associated with a poor prognosis. Sirt1, secreted by most Reed–Sternberg cells may decrease T-reg function [29]. Indolamine 2,3-dioxygenase expressed by histiocytes, dendritic cells, and some endothelial cells in the microenvironment is an enzyme that suppresses T-cell immunity. It is most commonly detected in Hodgkin lymphoma patients with advanced stage of disease and high levels of expression are associated with a poor prognosis [30]. A proportion of tumor infiltrating macrophages >25% was found to confer an unfavorable prognosis on patients with stage I-II Hodgkin lymphoma [31]. Maturation and differentiation of macrophages is dependent upon CSF1R interactions with its ligands. This pathway is activated in classical Hodgkin lymphoma, although CSF1R+ tumor associated macrophages are uncommon in Hodgkin lymphoma [32]. IL13 production by Reed–Sternberg cells appears to facilitate fibrosis commonly seen in lymphoma tissue from patients with nodular sclerosis Hodgkin lymphoma [33]. PRMD1, a major regulator of plasma cell differentiation, levels are low in Hodgkin lymphoma tissue. FOXO, which is important in normal B-cell development, acts as a tumor suppressor in classical Hodgkin lymphoma and upregulates PRMD1, which also acts as a tumor suppressor [34].

Many genes that have been implicated in the pathogenesis of Hodgkin lymphoma include polymorphisms of toll-like receptor genes and certain haplotypes of the myeloid differentiation primary response protein 88 gene (MYD82) [35] which are found more frequently in tissue from Hodgkin lymphoma patients than normal controls. To date, more than 35 genes have been implicated in the development of Hodgkin lymphoma and testing for the expression of a dozen or so of them by RT-PCR on paraffin-embedded formalin-fixed biopsy tissue has allowed for the prediction of 5-year freedom from progression with some accuracy [36]. Clinical outcome in advanced classical Hodgkin lymphoma is associated with a specific miRNA signature (MIR21, MIR30D, and MIR92B) which is associated with pathogenesis and therapeutic resistance [37]. PRDX2, a tumor suppressor gene, is methylated and transcriptionally silenced in Reed–Sternberg cells as it is in the cells of several other hematologic malignancies [38]. TNFRSF14, another tumor suppressor gene, is downregulated in Reed–Sternberg cells of a large minority of Hodgkin lymphoma patients, which may contribute to the pathogenesis of the disease [39]. The detection of markers of cellular senescence such as p16 (INK4a) and p21 (CIP1/WAF1) in >30% of Reed–Sternberg cells appears to predict for improved event-free survival [40].

Genetic susceptibility for Hodgkin lymphoma has been associated with a number of loci, including 2p16, 5q31, 6p31, 8q24, 10p14, and, more recently, 19p13.3 [41]. In a study of 259 genes on formalin-fixed paraffin-embedded tissue, a 23 gene outcome predictor superior to the International Prognostic Index was identified that revealed patients at increased risk of death when treated with standard chemotherapy regimens [42]. Alterations of chromosome 9p24 in classical Hodgkin lymphoma activates the programmed death 1 (PD-1) ligand pathway. This activation correlates with increased numbers of CD68+ macrophages, increased PD-1 expression in the peritumor microenvironment, and decreased numbers of FoxP3+ T-cells, which leads to immune escape [43]. Progression-free survival was significantly shorter for patients with 9p24.1 amplification

and enhanced PD-1 expression, in patients with advanced disease and early-stage disease in recent studies [44, 45]. By mapping transcription factor motifs, high levels of interferon regulatory factor (IRF5) were identified in Hodgkin lymphoma cells and found to be crucial for their survival [46].

Paietta et al. described an ectosialyltransferase on the surface of cultured Hodgkin lymphoma cells that agglutinates lymphocytes and may serve as an immunomodulator contributing to the immunodeficiency associated with Hodgkin lymphoma [47–52]. The same cell line expresses both CSF-1 and cfms, which suggests that Hodgkin cells may be derived from the monocyte/macrophage lineage [53].

Recently it was demonstrated that hypoxia induces Reed–Sternberg cell characteristics in mature B-cells and it was proposed that hypoxic conditions in the germinal center may initiate differentiation of B-cells toward a Reed–Sternberg cell phenotype [54] and genome-wide association studies identified SNPs and HLA alleles common to Hodgkin lymphoma and multiple sclerosis, two diseases that share a number of epidemiological characteristics [55].

More detailed information on the etiology and pathogenesis of Hodgkin lymphoma is presented in other chapters in this section, and an excellent review of lymphomagenesis in Hodgkin lymphoma has recently appeared [56].

Patterns of Disease

Initial Presentation

Nearly all patients with Hodgkin lymphoma present with asymptomatic lymphadenopathy. The apparent onset of the disease occurs predominantly in the cervical–supraclavicular nodes (60–80%), and less commonly in axillary nodes or inguinal nodes (10–20%). In some cases the first manifestations of disease are cough or dyspnea related to mediastinal lymphadenopathy; in other cases they consist of systemic symptoms (fever, night sweats, or weight loss) with or without apparent superficial lymphadenopathy. Pruritus, which occurs only with nodular sclerosis histology, is a symptom with no prognostic significance. In rare cases a mediastinal mass is found by routine X-ray as the first evidence of lymphoma.

In most patients the pattern of lymph node spread is central (cervical, mediastinal, para-aortic). For reasons that are not yet clear, certain chains of lymph nodes (mesenteric, hepatic hilar, hypogastric, presacral, epitrochlear, popliteal) are seldom or rarely involved. Pulmonary hilar lymph nodes are clinically affected in about one-fifth of patients with mediastinal adenopathy, and they are more frequently involved when a large mediastinal mass is present. Bulky disease is defined as a neoplastic mediastinal mass > one-third the transverse diameter of the thorax at the level of T5 or T6 on a standing posteroanterior chest radiograph [57, 58], whereas in other sites it is defined as any mass with a diameter over 10 cm. Patients with bulky mediastinal involvement represent approximately 25% of all cases and may have extension of the tumor into lung, pericardium, or chest wall. Systemic symptoms are also often present. Rarely, extranodal Hodgkin lymphoma is primary in virtually any anatomic site, including lung, any part of the gastrointestinal system including liver, bone [59] and brain [60] which is more frequent in patients infected with HIV and may be more common in familial Hodgkin's lymphoma [61], and an occasional patient will present with backpain due to spinal compression from an epidural mass of lymphoma [62] or skin involvement [63] which can often be traced to regional lymphatic blockage from an enlarged lymph node and retrograde flow of tumor cells to the skin, and digital clubbing, which is occasionally associated with cavitating lung lesions of Hodgkin lymphoma [64–66].

Limbic encephalitis and cerebellar degeneration rarely complicate Hodgkin lymphoma but do not occur in non-Hodgkin lymphoma [67]. Rarely, patients present with or have a history of immune thrombocytopenic purpura or hemolytic anemia [68] and have a history of rheumatoid arthritis more frequently than expected [69]. In addition, patients with a history of certain chronic infections (sinusitis, tuberculosis, encephalitis, herpes zoster) may be at increased risk for Hodgkin lymphoma [70].

Vanishing bile duct syndrome is diagnosed rarely at presentation in Hodgkin lymphoma patients. Only about 25 cases have been recorded in the literature. It is characterized by cholestasis and progressive loss of interlobular bile ducts in more than 50% of portal tracts in patients who do not have infiltration of the liver with lymphoma. It may be caused by cytokine release from tumor cells or by an immunologic mechanism. Patients usually present with leukocytosis, transaminase and alkaline phosphatase elevations and direct hyperbilirubinemia, but the diagnosis must be made by liver biopsy. Many cases will resolve with full-dose chemotherapy for the lymphoma, but some cases have been fatal [71, 72].

Approximately 1 of every 200 patients with chronic lymphocytic leukemia will have a transformation to Hodgkin lymphoma within 10 years (Richter transformation) [73]. This transformation to Hodgkin lymphoma has a poorer prognosis for response to therapy and survival compared with de novo Hodgkin lymphoma [73–75].

The risk for developing Hodgkin lymphoma in HIV-infected individuals is 5–25 times greater than that of the general population [76] and may be increasing. With the advent of more effective HIV therapy, the risk of non-Hodgkin lymphoma has decreased and the risk of Hodgkin lymphoma has increased. One interpretation of this observation is that both lymphomas are etiologically related

but are expressed differently depending on the degree of host immunodeficiency. This suggestion is consistent with the observation of families with both lymphomas in the pedigree [77]. It seems clear from many studies [78–80] that HIV+ patients with classical Hodgkin lymphoma have the same outcome from proper treatment as do HIV- negative patients.

In the great majority of patients, the initial pattern of spread occurs predictably via lymphatic channels to contiguous lymph-node chains and other lymphatic structures. This observation has provided the basis for prophylactic irradiation of adjacent nodal chains in patients with apparently localized nodal disease eligible for treatment by radiotherapy alone [81, 82].

The pattern of disease presentation is related to the histopathologic subgroup. Most patients with lymphocyte predominant (LP) histopathology present initially with only peripheral sites involved (cervical, inguinal nodes) and are most often male patients with stage I disease. Nodular sclerosis (NS) patients usually present with supradiaphragmatic disease and mediastinal node involvement and are often females with stage II disease. In patients with one of these variants, limited extension of supradiaphragmatic disease and presence of systemic symptoms, or occult involvement elsewhere is extremely rare. Patients with mixed-cellularity (MC) histology usually present either with upper torso disease or with lymphoma on both sides of the diaphragm (stage III). Patients with lymphocyte-depleted (LD) histology are usually stage III or IV, elderly, or relapsed.

Vascular invasion, which is noted in about 20% of autopsy cases, appears most often in LD histology and in advanced phases of disease [83]. Most extranodal sites manifest a markedly greater degree of vascular invasion, which suggests that vascular invasion is a factor in extranodal spread and in lethal cases of Hodgkin's disease [83]. Spleen, liver, lung, and bone marrow are the most frequent extranodal sites involved. Bone marrow is only likely to be involved in advanced stages of disease with or without systemic symptoms. Liver invasion is always associated with spleen involvement. Often, extranodal sites are involved by direct extension from enlarged adjacent nodes, as is the case with lung, pleural, or pericardial involvement which almost always is due to contiguous spread from bulky mediastinal lymphadenopathy.

Unexplained histologic lesions such as non-caseating epithelioid granulomas in liver, spleen, and/or lymph nodes of patients with any stage of disease are frequently found [84]. These lesions do not impair prognosis if left untreated, and therefore are unlikely to represent early lymphoma. They may represent a host response to the lymphoma, but this is unproven.

Elevated hepatic enzyme levels, including alkaline phosphatase, do not always signal liver involvement with Hodgkin's lymphoma [85]. Hepatic enzyme levels tend to correlate directly with stage of lymphoma whether the liver is involved with lymphoma or not.

The pathologic findings at autopsy have changed with modern therapy, including a reduction in the extent of disease at the time of death [86]. On occasion, Hodgkin lymphoma is first diagnosed at autopsy, especially in elderly patients (14% of cases in one series) [87]. The cause of death in one large autopsy series was progressive disease in 37%, infection in 43%, and miscellaneous causes in 20% [88].

A unique form of Hodgkin's lymphoma designated nodular, lymphocyte predominant Hodgkin's lymphoma deserves special consideration. It is relatively uncommon and almost always presents as stage I or II. The median age of patients is approximately 40 years, most patients are male (except Black patients who are approximately 50% female [89]), and only about 6% have B symptoms [90]. Its histology is unique in that the Reed–Sternberg cells have multiple large vacuoles, giving them a "popcorn" appearance. Those cells express CD20 but lack expression of CD15 and CD30, which are expressed by Reed–Sternberg cells in classical Hodgkin's lymphoma [91, 92]. A minority of patients have a variant histology characterized by the presence of lymphoma cells outside the B-cell nodules and/or a B-cell depleted microenvironment [93]. Such patients have a poorer prognosis with standard therapy than those with typical histology [93]. A minority of patients approaching 17% will transform over a 30 year period to a diffuse large B-cell lymphoma [94, 95]. Risk factors for transformation include prior chemotherapy and splenic involvement [95]. If treated properly, transformation does not impair overall survival compared with patients who do not transform [95]. Mutated SGK1, DUSP2, and JUNB have recently been identified as novel key players in the pathogenesis of nodular lymphocyte predominant Hodgkin lymphoma [96]. Curiously, there is a high familial risk for nodular lymphocyte predominant Hodgkin lymphoma in first-degree relatives of patients, approximately threefold higher than for classical Hodgkin lymphoma, especially for female relatives of young patients [97]. Treatment recommendations for nodular lymphocyte predominant Hodgkin lymphoma have ranged from surgical excision alone, watch and wait, radiation alone, chemotherapy alone, combined-modality therapy, and rituximab with or without standard chemotherapy, depending on presentation [98]. In stage IA patients, all seem to result in similar excellent results in adults, except rituximab alone is associated with increased risk of relapse [99, 100]. In pediatric low-risk patients with early-stage disease, surgical excision alone may be all that is necessary for the majority of patients and less intensive chemotherapy without radiation therapy is recommended for the rest [101].

Staging Procedures and Prognostic Estimate

Staging Systems by Prognostic Factors

Criteria for staging of Hodgkin's lymphoma were defined at the Rye (1965), Ann Arbor, and Cotswolds (1970–1989) conferences [102, 103], and are still universally applied worldwide. The staging system takes into account the involvement of one or more nodal areas on the same (stage I–II) or both sides of the diaphragm (stage III), of extranodal sites contiguous (E suffix) or noncontiguous with nodal involvement (disseminated involvement), of extranodal organ or tissue (stage IV) involvement, and the absence or presence of systemic symptoms (fever and/or night sweats and/or unexplained loss of more than 10% of body weight within the preceding 6 months) to apply suffix A or B, respectively, to any disease stage.

The Cotswolds Committee stated a number of classification criteria based on recent better knowledge of the natural history and treatment of the disease due to the advent of modern imaging techniques. It was particularly recommended that (1) CT scanning (now, Pet-CT) be included as a technique for evaluating intrathoracic and infradiaphragmatic lymph nodes; (2) the criteria for clinical involvement of the spleen and liver be modified to include evidence of focal defects by two imaging techniques and that abnormalities of liver function be ignored; (3) the suffix X be introduced to designate bulky disease (no longer used); and (4) a new category of response to therapy be established termed unconfirmed/uncertain complete remission (CRu), to accommodate the difficulty of assessing persistent radiologic abnormalities of uncertain significance following primary therapy. Table 43.1 shows the Rye–Ann Arbor–Cotswolds staging system as currently applied.

In 1998 an International Prognostic Factor Project on advanced Hodgkin's disease was developed based on data from 1618 patients treated at 25 centers [104]. The prognostic score was defined as the number of adverse prognostic factors at diagnosis (Table 43.2).

Certain blood tests and biopsy examinations have been more recently described as indicators of prognosis and not evaluated in the international prognostic score. Serum ferritin levels correlate with stage of disease [106] and prognosis [107, 108]. In a recent study, serum ferritin levels >350 μg/L were associated with a lower complete remission rate, progression-free survival and overall survival than observed in patients with lower ferritin levels [107]. CD34+ peripheral blood cells in a concentration >0.0045 × 10^9/L predicted a reduced progression-free survival in a study of 60 newly diagnosed patients [109]. The CD68+ count in biopsy specimens inversely correlated with progression-free survival [110]. Detection of circulating cell-free DNA with genomic imbalances such as gain of chromosomes 2p and 9p has been proposed as a biomarker of Hodgkin lymphoma that may be useful in prognostication [111]. Two studies have shown that a low absolute lymphocyte count to absolute monocyte count ratio in peripheral blood has prognostic significance in Hodgkin lymphoma [112, 113]. A low ratio predicts for poor event-free and overall survival. High concentrations of myeloid-derived suppressor cells (CD66b + CD33dim HLA-DR) correlate with unfavorable international prognostic index scores and a shorter progression-free survival [114].

Table 43.1 The Rye–Ann Arbor–Cotswolds staging

Classification
Stage I
Involvement of a single lymph node region or lymphoid structure (e.g., spleen, thymus, Waldeyer's ring) or involvement of a single extralymphatic site (IE)
Stage II
Involvement of two or more lymph node regions on the same side of the diaphragm (hilar nodes, when involved on both sides, constitute stage II disease): localized contiguous involvement of only one extranodal organ or site and lymph node region(s) on the same side of the diaphragm (IIE): the number of anatomic regions involved should be indicated by a subscript (e.g., II_3)
Stage III
Involvement of lymph node regions on both sides of the diaphragm (III), which may also be accompanied by involvement of the spleen (III_s) or by localized contiguous involvement of only one extranodal organ site (IIIE) or both (III_s E) III1: with or without involvement of splenic, hilar, celiac, or portal nodes
III2: with involvement of para-aortic, iliac, or mesenteric nodes
Stage IV
Diffuse or disseminated involvement of one or more extranodal organs or tissues, with or without associated lymph node involvement

Designations applicable to any disease stage:
A: No symptoms
B: Fever (temperature above 38 °C), drenching night sweats, unexplained loss of more than 10% of body weight within the preceding 6 months
E: Involvement of a single extranodal site that is contiguous or proximal to the known nodal site

Recommended Pretreatment Evaluation

Table 43.3 outlines the procedures deemed necessary for correct staging of Hodgkin lymphoma [115]. An adequate surgical biopsy, possibly of more than one intact lymph node, should be undertaken for pathological examination. Inguinal nodes should not be biopsied if other equally suspicious peripheral nodes are present. When the diagnosis of Hodgkin lymphoma is made from biopsy of an extranodal site, a concomitant nodal biopsy for confirmation of diagnosis is desirable.

A detailed history must be obtained with information about the presence or absence of unexplained fever and its duration,

Table 43.2 The International prognostic score for advanced stages (IPS-7)

Independent variable with negative prognostic significance			
Variable	Value		
Albumin	<4 g/dL		
Hemoglobin	<10.5 g/dL		
Sex	Male		
Age	≥45 years		
Stage	IV		
Leukocytosis	White blood cells >15,000/mm^3		
Lymphocytopenia	Absolute lymphocyte count <600/mm^3 or <8% total white blood count		
Rates of freedom from progression and overall survival at 5 years according to individual and grouped prognostic scores			
Prognostic score	*N* patients (%)	Rate of FFP (%)	Rate of OS (%)
Single negative factors			
0	115 (7)	84 ± 4	89 ± 2
1	360 (22)	77 ± 3	90 ± 2
2	464 (29)	67 ± 2	81 ± 2
3	378 (23)	60 ± 3	78 ± 3
4	190 (12)	51 ± 4	61 ± 4
5–7	111 (7)	42 ± 5	56 ± 5
Grouped negative factors			
0 or 1	475 (29)	79 ± 2	90 ± 2
2 or more	1143 (71)	60 ± 2	74 ± 2
0 to 2	939 (58)	74 ± 2	86 ± 2
3 or more	679 (42)	55 ± 2	70 ± 2
0 to 3	1317 (81)	70 ± 2	83 ± 1
4 or more	301 (19)	47 ± 2	59 ± 2

FFP freedom from progression, *OS* overall survival.
Data from Hasenclever D, Diehl V. A prognostic score for advanced Hodgkin's disease. N Engl J Med. 1998;339:1506. Recent data suggest that consideration of age, stage and hemoglobin level may provide a simpler and more accurate prognosis [105]

Table 43.3 Recommended procedures for proper staging

Recommended procedures
Adequate surgical biopsy reviewed by an experienced hemopathologist: in primary extranodal lymphomas, biopsy should also include a palpable lymph node when palpable
Detailed history with special attention to the presence or absence of systemic symptoms
Careful physical examination, emphasizing node chains, size of liver and spleen, Waldeyer's ring inspection, and bony tenderness
Routine laboratory tests: complete blood count, erythrocyte sedimentation rate, liver function tests, serum uric acid, serum lactate dehydrogenase
Whole-body PET-CT scan
Unilateral bone marrow biopsy in stages III–IV and any stage with B symptoms
Cytologic examination of any effusion
Any further study suggested by clinical symptoms or signs

unexplained sweating (especially at night) and its severity, unexplained weight loss as a percentage of usual body weight and rapidity of loss, and pruritus with its extent and severity. The presence of alcohol-induced pain, peculiar to Hodgkin lymphoma patients, a family history of Hodgkin lymphoma [116] or other hematologic malignancy [77, 117], any history of immunosuppressive illness, such as infection with human immunodeficiency virus (HIV), or EBV infection, such as mononucleosis or a previous history of neoplasm together with any previous chemotherapy or radiation therapy should also be documented. A careful and complete physical examination must be performed with special attention to the number of enlarged lymph nodes and their dimensions, presence or absence of enlarged liver or spleen involvement, and Waldeyer's ring involvement. A complete laboratory workup is required. A whole body PET-CT scan prior to any therapy should be performed [118]. Radiolabeled fluorodeoxyglucose positron emission tomography (^{18}FDG-PET) has emerged as a very useful tool in evaluating the presence of active neoplastic tissue in residual masses after therapy, especially in the infradiaphragmatic areas. By utilizing the ^{18}FDG metabolism of neoplastic cells, ^{18}FDG-PET scanning seems to be able to recognize small lesions (less than 1 cm in diameter), with a high sensitivity approaching 100% despite a low specificity (slightly superior to 70%). PET scanning may reveal involved areas undetectable with other imaging techniques [119]. It should be noted, however, false-positive PET scans are not uncommon [120]. This is particularly true for bone marrow, where a positive PET scan may be due to red marrow hyperplasia due to anemia in patients with advanced lymphoma [121]. Furthermore, in some studies interim PET scans have not been predictive of outcome [122] and inter-reader reliability has been poor in others [123]. Nevertheless, PET-CT is currently the standard radiographic method for staging Hodgkin lymphoma [118, 124].

Evaluation of Response and Follow-Up Studies

After completion of planned therapy, response should be documented on the basis of clinical findings, and the results of a PET-CT scan supplemented by tissue biopsies if necessary. A number of patients have residual abnormalities after therapy, typically mediastinal widening. It is well known that residual non-PET avid masses, especially in the mediastinum do not necessarily represent residual disease but rather may indicate necrosis or fibrosis, and abnormal radiographic findings may persist for months to several years in such patients who are otherwise without evidence of disease [125]. Within the bounds of acceptable morbidity, pathologic

examination such as mediastinoscopy may be appropriate, although the difficulties of sampling artifacts should be kept in mind.

It is becoming commonplace to obtain a second PET-CT scan after two or three cycles of therapy to assess treatment progress if it cannot be fully assessed by physical examination alone. In this scenario, treatment may be reduced in intensity, i.e., omit radiation therapy after chemotherapy for early-stage patients if the repeat scan is negative [126], or it may be intensified if resolution of disease is slow or not apparent, by intensifying chemotherapy or adding radiation therapy. This approach at present seems valid for early-stage patients (some authorities disagree), but most studies of advanced-stage patients do not have long enough follow-up data to fully evaluate the results [127–134].

After completion of therapy, it is recommended that patients be seen at 3 month intervals during the first and second posttreatment years, at 4–6 month intervals from the 3rd to 5th year, and annually thereafter. The frequency and type of follow-up studies depend on and should focus on the initial sites of disease, as well as the detection of medium and long-term sequelae of treatment. Appropriate workup should be initiated when symptoms or signs of possible recurrent disease are noted. Because of the relatively high incidence of second neoplasms, investigations useful for the early detection of new malignancies, particularly breast and lung cancer should be part of the follow-up studies for patients in remission [135, 136]. Screening mammography or breast ultrasound, even in young women treated by mantle-field irradiation, is an example of this policy [136] and one should be mindful of the relatively high risk of cardiac complications of treatment. However, routine PET/CT or CT imaging for patients in complete remission is not recommended because yields are extremely low [137–139].

Treatment

Evolution of the Treatment Strategy

Treatment of Hodgkin's lymphoma has continuously improved since the 1960s and 1970s, and today 90% or more of all patients can be cured by first- or second-line therapeutic strategies [140]. Hodgkin lymphoma represents a remarkable example of how progress in clinical research can, occasionally, outpace progress in basic research (Table 43.4).

Radiation therapy progressively developed following the work of Gilbert (1939) and Peters (1950) and became a

Table 43.4 Chronologic flow of major concepts and events influencing treatment evolution of Hodgkin lymphoma

Investigator(s) (years)	Major concepts and events
Gilbert (1925–1939)	Concept of destruction of all lesions with the first course of radiation therapy: segmental irradiation to encompass suspected microscopic disease
Peters (1950–1958)	Improved 5- and 10-year survival by prophylactic irradiation of adjacent lymphoid areas: first three-stage clinical classification
Kinmoth (1952)	Lower extremity lymphangiography
Easson and Russel (1963)	Concept of cure by radiation therapy
Lukes (1963–1964)	Relationship of histologic features to clinical stages and prognosis
Kaplan (1962–1965)	Development of wide-field technique with radiation in continuity with multiple-node chains (mantle, inverted Y, and total lymphoid radiation therapy): identification of tumoricidal dose levels
Rosenberg and Kaplan (1965)	Evidence for an orderly progression in the spread of Hodgkin's disease
Lacher and Durant (1965)	Increased complete remission rate with vinblastine plus chlorambucil compared with single agents
Frei (1966)	Efficacy of a cyclic four-drug combination (MOPP)
Kaplan and Glatstein (1969)	Staging laparotomy and further studies on the pattern of anatomic distribution
DeVita (1970–1980)	Concept of high cure rate in advanced disease with MOPP
DeVita, Young (1971–1973)	No advantage of maintenance chemotherapy in complete responders
Rosenberg (1968–1981)	Trials with combined radiation therapy and chemotherapy, especially MOPP
Wiernik (1968–1985)	Trials of combined-modality therapy with MOPP and radiation therapy
Bonadonna and Santoro (1973–1987)	Development of non-cross-resistant chemotherapy (ABVD)
Wiernik (1976)	Randomized trials of MOPP versus combined-modality therapy for early-stage disease
Longo et al., Cimino et al. (1982–1990)	Randomized trials of MOPP versus radiation therapy alone for early-stage Hodgkin's disease
Canellos, Connors, Viviani et al. (1980–1998)	Randomized trials comparing MOPP, ABVD, and hybrid or alternating MOPP/ABVD in advanced stage
Santoro et al., Horning et al. (1990–1997)	Low-toxic brief chemotherapy (ABVD, VBM) and involved-field radiotherapy for early-stage Hodgkin's disease

(continued)

Table 43.4 (continued)

Investigator(s) (years)	Major concepts and events
Horning, Chopra, Sweetenham Josting et al. (1990–2000)	Studies with high-dose chemotherapy and bone marrow transplantation for salvage therapy
Diehl, Horning, Wiernik (1987, 1995, 2006)	New-generation intensive regimens (BEACOPP, Stanford V, MVC) for advanced stages and salvage therapy
Younes (2010)	Brentuximab vedotin major activity in advanced disease
Ansell (2015)	Nivolumab major activity in advanced disease

major treatment for Hodgkin lymphoma with the beginning of the megavoltage era. Studies by Kaplan and the Stanford group [81] led to the development and refinement of many concepts and techniques that constitute modern radiation therapy for lymphomas and particularly for Hodgkin lymphoma [141–144]. Until the end of the 1960s, radiation therapy was the only successful therapeutic tool that, under appropriate circumstances, was able to achieve cure in a sizeable fraction of patients with Hodgkin lymphoma. Chemotherapy was developed after the end of World War II, and lymphomas and leukemias were among the first neoplasms shown to respond to drug treatment. Although the great majority of available cytotoxic agents can produce objective responses in Hodgkin lymphoma, it was only in 1970 with the four-drug regimen known as MOPP (mechlorethamine, vincristine, procarbazine, and prednisone) that DeVita and Carbone documented that chemotherapy could induce durable complete remissions and cure with high frequency in advanced Hodgkin lymphoma [145].

In the late 1970s and 1980s, radiation therapy and chemotherapy were sequentially combined for the treatment of early as well as advanced stages to maximize relapse-free survival (RFS), and to improve the cure rate. In an attempt to eradicate both MOPP-sensitive and MOPP-resistant neoplastic cells, Bonadonna et al. designed the ABVD (doxorubicin [Adriamycin], bleomycin, vinblastine, dacarbazine) regimen and mounted a number of prospective trials that proved its effectiveness as a non-cross-resistant regimen to MOPP [146–151]. Further attempts to improve the cure rate took into consideration the Goldie–Coldmann hypothesis [152] by testing the effect of early introduction of as many effective drugs as possible in treatment strategies. Trials from the Milan, Vancouver, and other groups compared alternating MOPP/ABVD with the so-called hybrid regimen which alternates half MOPP and half ABVD [153–155]. The hybrid regimen or MOPP alternating with ABVD has not demonstrated any real advantage over ABVD alone [156].

In 1992 Canellos and colleagues from the Cancer and Leukemia Group B (CALGB) confirmed in a large randomized study that ABVD alone was superior to MOPP. Since then, ABVD chemotherapy has become the gold standard against which all newer regimens should be tested [157].

In the 1990s, further efforts to improve the outcome of patients with Hodgkin lymphoma have consisted of the intensification of primary chemotherapy with hematopoietic colony-stimulating factor (CSF) support, with the aim both to increase complete remission rate and to minimize late toxic effects. A major example is the BEACOPP regimen (bleomycin, etoposide, doxorubicin, cyclophosphamide, vincristine, procarbazine, prednisone) [157, 158], designed by Diehl and coworkers from the German Hodgkin's Study Group, as well as of the Stanford V regimen of Horning et al. (weekly alternation of doxorubicin, vinblastine, mecloretamine, vincristine, bleomycin, etoposide, plus prednisone) [159, 160]. Recently, it was demonstrated that the Stanford V regimen is not superior to ABVD in any outcome measure [161], nor is BEACOPP.

The question of whether a clinical dose–response effect can be exploited with the use of high-dose chemotherapy with autologous [162–167] and allogeneic [168–170] stem cell rescue has been tested worldwide as salvage therapy for relapsed or refractory patients. Since initial attempts in the early 1980s, numerous investigators used high-dose chemotherapy with autologous bone marrow transplantation (ABMT) or peripheral blood stem cell transplantation (PBSCT) for salvage treatment of patients with relapsed Hodgkin's disease. An early publication of a randomized comparison between high-dose and standard-dose chemotherapy [167], demonstrated that a proportion of patients could be cured after relapse with this high-dose strategy. Thus, the trend toward a better outcome for patients treated by high-dose chemotherapy for relapse, along with the low morbidity rate, now reported as <1–4% for autologous and 10–20% for allogeneic transplants confirms the validity of these approaches.

Improvement in treatment results has been achieved in all stages of the disease. Today, no stage is beyond cure when treated appropriately at the time of diagnosis or when treated for recurrence. New agents such as brentuzumab vedotin and nivolumab seem destined to increase the number of disease-free long-term survivors of Hodgkin lymphoma even further. This represents a dramatic improvement in the overall prognosis of a disease that 40 years ago was considered to be almost universally fatal.

General Treatment Strategy

Careful patient selection remains important to identify those who will benefit from each treatment strategy, especially concerning the multimodality approach, such as the following:

1. In early stages (I, IIA), the multidisciplinary approach is now considered standard, but is seriously challenged by data on chemotherapy alone [171–179]. As the vast majority of these patients (approximately 90%) can be cured, the choice of treatment is presently aimed at reducing both acute and late toxicities by introducing less toxic drugs and reducing the dose and extension of radiation fields, or eliminating radiation therapy altogether, especially in children.
2. In patients presenting with massive mediastinal involvement, the role of combined-modality therapy is well established in all stages of disease [177].
3. In patients with more advanced stages of disease (IIA$_2$, IIB, III–IV A and B), the percentage of nodal relapses is about 20% even after effective chemotherapy [180–183].

The usefulness of consolidation radiotherapy to all sites of disease has long been a matter of debate. Some groups prefer to irradiate all nodal sites of disease at diagnosis even if the advantage of this approach is not fully demonstrated [179]. It is likely that irradiating all nodal areas in a patient with extensive stage IV disease will not enhance the probability of cure, whereas radiotherapy may be advantageous in those patients with advanced, exclusively nodal disease, but not more effective than continuing chemotherapy.

Although improved in the last 25 years, salvage treatment is curative only in about one-half of relapsed patients, except under the most favorable circumstances, such as limited-stage relapse beyond the 12th month of initial complete remission [182, 183]. As pointed out by Rosenberg [184] new salvage therapies should not be evaluated hastily since curability and comparisons of toxicities require 5–10 years of careful evaluation.

Nearly one-third of patients with Hodgkin's lymphoma die without evidence of lymphoma at autopsy, and in a significant number of patients, death is attributable to complications of therapy [184].

Therefore, treatment programs should be designed not only to improve therapeutic results but prevent or reduce iatrogenic complications such as cardiac and neoplastic as well. Long-term complications of treatment are discussed next.

Treatment of Early Stages (IA–B, IIA)

The vast majority of patients with early-stage Hodgkin's disease (IA–IIA) can be cured by currently available therapeutic options. Thus, a major aim of treatment is to limit medium- and long-term toxic effects rather than to further increase the cure rate. However, it should be noted that there is no complete agreement on the definition of early stages, as some authors include cases with bulky disease or B symptoms, whereas others do not (Table 43.5). As a consequence, it is somewhat difficult to draw precise conclusions from published series concerning the same treatment modalities but with different patient selection criteria.

However, it seems clear that radiation therapy alone or chemotherapy alone can cure over 90% of patients with Stage IA or IIA without bulky mediastinal disease, and that patients with bulky mediastinal disease require combined-modality therapy for optimal results.

Table 43.5 Prognostic subgroups in stage I–II

EORTC	
Very favorable	CS IA, female sex, age < 40, and LP histology, or NS histology and nonbulky mediastinum
Favorable	Cases classifiable neither as very favorable nor as unfavorable
Unfavorable	One of the following factors: bulky mediastinum, stage A with ESR >50, B with ESR >30, age > 50, more than four sites involved
GHSG	
Unfavorable	Bulky mediastinum or elevated ESR, or
	• Three involved sites, or
	• Disseminated extranodal or splenic involvement
Favorable	All others
Stanford	
Very favorable[a]	CS I and female sex, or
	CS I mediastinum, or
	CS I and male sex with LP histology, or
	CS II, female sex, age < 27, and <three involved sites
Harvard	
Very favorable	CS IA and female sex, or
	CS IA and male sex with LP histology, or
	CS IA and male sex with peripheral cervical site

GHSG German Hodgkin's Study Group, *CS* clinical stage, *LP* Lymphocyte-Predominant, *NS* nodular sclerosis, *ESR* erythrocyte sedimentation rate

[a]Treated with mantle-field irradiation alone, non-laparotomy staged

Radiotherapy

Up to the 1980s, the standard approach to stage IA–IIA Hodgkin's disease included staging laparotomy and splenectomy and, if these procedures are negative, mantle-field irradiation at therapeutic doses (36–44 Gy), followed by irradiation of para-aortic nodes and the splenic pedicle (subtotal nodal irradiation, STNI) at lower doses (30–36 Gy). With this approach, up to 90% of patients were alive at 10 years with a freedom from progression of 70–80% in one study [185]. Relapsing patients could, in fact, obtain second durable remissions by salvage chemotherapy. This approach has been reconsidered by a number of research groups. First, many radiation therapists documented a relapse rate in stage

IIA that was at least double that initially reported, and in stage IIB patients a failure rate of about 50% was observed with radiation alone [171, 186, 187]. However, it should be pointed out that selection criteria often included the so-called unfavorable early stages as outlined by the European Organization for Research and Treatment of Cancer (EORTC): MC and LD histologies, male sex, age older than 40 years, erythrocyte sedimentation rate more than 70, and mediastinal mass. In the presence of one or more unfavorable features, patients treated with extensive radiation therapy alone had a 6-year RFS of 53% versus nearly 100% when treated with combined-modality therapy [187]. Furthermore, the Stanford group tested subtotal nodal irradiation versus involved-field irradiation plus six cycles of MOP(P) in pathological stages IA–IIA, and concluded that adjuvant chemotherapy could replace radiation of occult disease [187]. The results of a number of major trials comparing radiotherapy, chemotherapy, and combined-modality therapy are shown in Table 43.6.

Over the last decade, indications for radiotherapy in early-stage Hodgkin's disease have been progressively revised. At present, only a limited-stage IA presentation with no adverse factors is considered to be appropriately treated with mantle-field or subtotal nodal irradiation alone. For all others, combined-modality treatment or chemotherapy alone is recommended [192–195]. Radiation therapy for Hodgkin lymphoma is discussed extensively in Chap. 46.

Chemotherapy

In the second half of the 1980s chemotherapy was introduced in the management of early-stage Hodgkin's lymphoma. Some groups have tested the efficacy of chemotherapy alone in this setting in order to eliminate late effects related to combined-modality therapy. Two groups published randomized comparisons between MOPP chemotherapy and radiotherapy, reaching opposite conclusions. The Italian group [196] found that radiation therapy was ineffective as salvage treatment after MOPP failure; thus in their opinion, radiation therapy remains the treatment of choice. The National Cancer Institute (NCI) group observed a superiority of MOPP chemotherapy over radiation therapy and concluded that MOPP was at least as effective as radiation therapy [172].

The National Cancer Institute of Canada and ECOG compared two courses of ABVD (doxorubicin, bleomycin, vinblastine and dacarbazine) followed by radiotherapy with four to six courses of ABVD alone. After a median of 4.2 years PFS was better for patients who received radiation therapy. Progression after ABVD alone was more frequent in sites that would have been irradiated in the other arm of the study, but there was no difference between the arms with respect to freedom from second progression or death rate [197]. Therefore, patients who progressed after initial treatment with ABVD alone could be successfully treated with salvage radiation therapy if required. BEACOPP (bleomycin, etoposide, doxorubicin, cyclophosphamide, vincristine, procarbazine, and prednisone) was developed as a chemotherapy regimen initially thought to be superior to ABVD. However, in several recent long-term follow-up studies ABVD and BEACOPP appear to yield equivalent results in terms of response rate, progression-free and overall survival. In fact in some but not all studies [198] overall survival is actually impaired by BEACOPP compared with ABVD due to an increased frequency of late developing second malignancies [199]. In summary, the longer the follow-up of comparative studies, the less difference in outcome between these two regimens is observed.

Studies have been conducted in an effort to determine whether both major drug regimens for Hodgkin lymphoma

Table 43.6 Randomized trials comparing combined modality versus radiotherapy or chemotherapy alone in early-stage Hodgkin's disease

Author (ref)	Regimen	RT	Stages	FFP (%)	OS (%)	Median FU (years)
Pavlovsky [188]	CVPP + RT vs. CVPP	IF	Unfavorable I–II	75	84	7
				34	66	
Horning [190]	VBM + RT vs. RT	IF	I–IIA, IIB, IIA favorable	95	95	3
		STNI/TNI		70	67	
Radford [189]	VAPEC-B + RT vs. RT	IF		91	NR	3.3
			I–IIA			
		STNI		73	NR	
Santoro [191]	ABVD/IF vs. ABVD/ STNI	IF		95	100	3
			I-IIA			
		STNI		94	100	
Wiernik [171]	MOPP + RT vs. RT	EF	IA, IIA, IIB, IIIA	94	91	5.8
				66	76	

RT radiotherapy, *FFP* freedom from progression, *OS* overall survival, *IF* involved fields, *EF* extended field, *FU* follow-up period, *STNI* subtotal nodal irradiation, *TNI* total nodal irradiation, *NR* not reported

Table 43.7 Common combination chemotherapy regimens for treatment of Hodgkin lymphoma

ABVD: All drugs administered IV on days 1 and 15 of a 28 day cycle at a dose of:	
Doxorubicin	25 mg/m^2
Bleomycin	10 units/m^2
Vinblastine	6 mg/m^2
Dacarbazine	375 mg/m^2
Maximum eight cycles total, including two cycles after complete remission.	
BEACOPP: All drugs administered on a 21 day cycle	
Bleomycin	10 units/m^2 IV day 8
Etoposide	100 mg/m^2 IV days 1–3
Doxorubicin	25 mg/m^2 IV day 1
Cyclophosphamide	650 mg/m^2 IV day 1
Vincristine	1.4 mg/m^2 day 8 (maximum dose, 2 mg)
Procarbazine	100 mg/m^2 PO days 1–7
Prednisone	40 mg/m^2 PO days 1–14
Maximum of eight cycles total, including two cycles after complete remission	

can be made less toxic in the short- and long-term while retaining efficacy. One such study determined that bleomycin and dacarbazine cannot be omitted from the ABVD in advanced-stage patients without significantly reducing its efficacy [200] while another study demonstrated that discontinuing bleomycin and/or vincristine because of drug-specific adverse events during treatment did not impair progression-free or overall survival in advanced-stage patients after BEACOPP treatment [201]. This information can probably be considered valid for early-stage patients as well (Table 43.7).

Most trials of the last 25 years have focused on the role of a combined chemotherapy–radiotherapy strategy in early-stage Hodgkin's disease. The debate arises from the evidence that salvage chemotherapy in patients relapsing after radiotherapy alone offers cure rates as high as initial combined-modality therapy. On the other hand, delivering chemotherapy after extensive radiotherapy results in a higher risk of second malignancies. At the Milan Cancer Institute, 116 patients with pathologically staged favorable presentation who were treated with extended-field radiotherapy were non-randomly compared with 85 patients with unfavorable presentations who were treated with MOPP (nitrogen mustard, vincristine, procarbazine, and prednisone) and radiotherapy. At 10 years, the freedom from progression was significantly different between stages I and II in the favorable group (85% vs. 59%), respectively, whereas stage lost its prognostic significance in the unfavorable group treated with combined-modality therapy [202]. Furthermore, in the favorable group a higher incidence of acute leukemia as a consequence of salvage chemotherapy was observed.

At Stanford, 67 patients with favorable pathological stages I and II (A and B), or IIIA were randomized between subtotal or total nodal irradiation alone or involved-field irradiation plus six cycles of the VBM regimen (vinblastine, bleomycin, methotrexate). At 5 years, freedom from progression was 70% after radiation alone and 95% after combined-modality treatment ($p < 0.0001$) [189]. In a subsequent trial, the authors focused on favorable clinical stages I and II: 78 patients were randomized to receive either extended-field irradiation (subtotal nodal) or involved-field irradiation after VBM. With a median follow-up of 4 years, 92% of the extended-field group and 87% of the combined-modality group were free of progression. Despite the fact that comparable results between the two groups would support the use of combined-modality therapy in all patients, the authors did not recommend its routine use outside of a clinical trial [190].

The Milan group compared two different radiotherapy strategies following primary chemotherapy by randomizing 103 patients to receive STNI or involved-field radiotherapy after four courses of ABVD. In a preliminary report, with a median follow-up period of 38 months, the response rate was 100%, freedom from progression was 95%, and survival was 100% without any difference between the two groups [191].

In a meta-analysis, the role of adjuvant chemotherapy was assessed in 13 randomized trials involving 3888 early-stage patients. The addition of chemotherapy to radiotherapy halved the 10-year risk of failure (16% vs. 33%), with a small statistically insignificant improvement in survival (79% vs. 76%). A reduction of Hodgkin's disease deaths of borderline significance (12.3% vs. 15.4%) was partly counterbalanced by an insignificant increase in deaths from other causes (12% vs. 10%) [144]. However, it could be argued that this meta-analysis refers to studies begun in the 1970s or early 1980s, when MOPP was the most frequently used combination. Thus, it is possible that less toxic regimens such as ABVD will reduce mortality from causes other than Hodgkin lymphoma and establish the combined-modality advantage. In another meta-analysis, Loeffler et al. [203] concluded that combined-modality therapy for advanced-stage disease resulted in inferior survival outcome compared with chemotherapy alone. In summary, at present the optimal initial treatment for early-stage Hodgkin's lymphoma is not known. Radiation alone, chemotherapy alone, or combined-modality therapy all have their advocates [204–206]. A reasonable approach is as follows: Stage IA: radiation therapy alone; Stage IIA or B with a large mediastinal mass: combined-modality therapy; All others: chemotherapy alone. Clearly, in young patients radiation therapy can be omitted if a PET/CT scan is negative for residual disease after two cycles of induction chemotherapy [207].

Nodular lymphocyte predominant Hodgkin's lymphoma patients need to be considered differently with respect to

treatment from classical Hodgkin's lymphoma patients. Limited-field radiotherapy is considered standard for early-stage disease (>90% of patients), since it has been shown to be as effective in all outcome measures as combined-modality therapy and more effective than chemotherapy alone [91, 208]. However, recent studies have demonstrated efficacy for rituximab in this Hodgkin's variant whose Reed–Sternberg cells are CD20+ [92]. New regimens that include rituximab are currently in clinical trial for this entity. However, as discussed above, surgical excision of stage I disease alone is frequently curative in this lymphoma, especially for pediatric patients.

Treatment of Advanced Stages

Stage IIB, III_2, and IV patients according to the Ann Arbor staging system are considered to be advanced. There is no consensus in the literature concerning stage III_1A, which is considered early stage by some investigators and advanced stage by others.

Conventional Approach

Primary chemotherapy is the standard approach for advanced stages. Up to the 1990s, the gold standard was the MOPP regimen [209], which induced complete remission in approximately 80% of patients. Of those, approximately 30% relapsed within 5–10 years.

MOPP is inferior to ABVD in the treatment of Hodgkin lymphoma. The first trial demonstrating the superiority of an ABVD-based regimen over MOPP was conducted at the Milan Cancer Institute in patients with stage IV disease. The alternating MOPP–ABVD regimen demonstrated a 15–20% advantage over MOPP [148]. Subsequently, the same group [150] demonstrated a significant advantage in terms of complete remission rate, freedom from progression, and RFS favoring ABVD (three courses followed by extended-field radiotherapy and three more courses) as compared with MOPP (same schedule) in 232 pathologically staged IIB and III patients. The comparable overall survival in the two groups was probably related to the fact that salvage ABVD was given to MOPP-resistant patients. The long-term results of this trial confirmed the efficacy of a combined-modality approach. As far as delayed toxicity is concerned, no signs of myocardial damage were seen in either arm. However, other studies with longer follow-up have reported a 10-year incidence of 5.5% cardiac disease-related hospitalizations after ABVD alone and 9.2% after ABVD plus mediastinal irradiation, compared with a 2.2% rate expected in the general population [210]. The ABVD group showed more pulmonary radiation fibrosis than the MOPP group, in particular after the last three cycles of bleomycin-containing chemotherapy. Permanent testicular failure occurred only after MOPP. Although MOPP was definitively more leukemogenic than ABVD, the frequency of solid tumors was about the same with the two combinations [211]. Clearly, the incorporation of alkylating agents (mechlorethamine and procarbazine) in the MOPP regimen is responsible for the increased incidence of sterility and leukemogenesis observed with that treatment.

In the CALGB study comparing MOPP, ABVD, and MOPP alternating with ABVD in 362 patients with advanced-stage disease ($IIIA_2$, IIIB, IV), 8-year freedom from progression was 37% for MOPP, 52% for ABVD, and 50% for alternating MOPP and ABVD. A similar difference in overall survival did not emerge. A 3% incidence of fatal febrile neutropenia in the MOPP group was counterbalanced by a similar incidence of fatal pulmonary toxicity with ABVD, and 1% of each after MOPP–ABVD [212].

Some groups have tested the alternating MOPP/ABVD with the so-called hybrid MOPP/ABVD, which consists of alternating half courses of MOPP with half courses of ABVD, with the aim of more rapid delivery of all active drugs in concert with the Goldie–Coldman hypothesis. In the Vancouver trial, alternating MOPP and ABVD was compared with hybrid MOPP–ABV, with ABV given on day 8 of each 28-day course. Both regimens produced excellent results, but the hybrid regimen was associated with a higher incidence of life-threatening febrile neutropenia and stomatitis, leading to a lowering of the upper age limit from 65 to 55 years in mid-study [153]. In 415 patients treated at the Milan Cancer Institute, alternating and hybrid programs (half MOPP and half ABVD every 14 days of each 28-day cycle) produced comparable results [154] in terms of both survival and acute toxicity.

The North American Intergroup [155], compared sequential MOPP followed by ABVD with hybrid MOPP/ABV in 737 patients with advanced-stage (III_2A, IIIB, IV) Hodgkin's disease with the aim of testing the Goldie–Coldman hypothesis by introducing doxorubicin late (sequential) or early (hybrid) in the therapeutic program. Patients assigned to the sequential schedule received six MOPP courses followed, if complete response was attained, by three ABVD courses. Patients in the hybrid group received MOPP–ABV as designed by Connors et al. [153]. Complete remission was achieved in 83% of cases on the hybrid and 75% in the sequential group ($p = 0.02$). After a median follow-up period of 7.3 years, the 8-year FFP was 64% for MOPP/ABV and 54% for MOPP → ABVD ($p = 0.01$). The 8-year OS rate was significantly better for MOPP/ABV as compared with MOPP → ABVD (79% vs. 71%, $p = 0.02$). Acute life-threatening (grade 4) or fatal (grade 5) toxicity was more frequent in the hybrid regimen. However, seven cases of acute myeloid leukemia and two of myelodysplasia were

Table 43.8 Therapeutic outcome at 5 or more years with MOPP, ABVD, or alternating/hybrid MOPP/ABVD in advanced-stage Hodgkin's disease

Regimens	No. of cases	Stage	FFP (%)	OS (%)
Milan [148, 150]	114		63	64
MOPP × 3 → RT → MOPP × 3		IIB, IIIA–B		
vs. ABVD × 3 → RT → ABVD × 3	118		81	71
MOPP	43		37	58
vs.		IV A–B		
MOPP/ABVD	45		61	69
MOPP/ABVD alternating	211	I–IIB, IIAX,	67	74
vs.		IIIA/B,		
MOPP/ABVD hybrid	204	IV A/B	69	72
CALGB [212]				
MOPP	123		37	66
vs. ABVD	123	$IIIA_2$, IIIB, IVA/B	52	73
vs.				
MOPP/ABVD	115		50	75
Vancouver [153]				
MOPP/ABV hybrid	153	IIIB, IVA, B	71	81
vs.				
MOPP/ABVD alternating	148		67	83
Intergroup [155]				
MOPP/ABVD alternating vs.	344	$IIIA_2$/B, IV A/B	54	71
MOPP/ABV hybrid	347		64	79

FFP freedom from progression, *OS* overall survival

diagnosed in patients receiving MOPP → ABVD as compared with one case of acute myeloid leukemia in the hybrid regimen ($p = 0.01$). Overall (Table 43.8), six to eight courses of ABVD alone has emerged as the gold standard in advanced-stage Hodgkin's disease, with which new-generation regimens should now be compared.

Newer Intensive Combinations

Optimization of dose intensity, inclusion of new active drugs, and the appropriate use of hematopoietic growth factors have been studied as methods by which the cure rate might be enhanced in patients with advanced Hodgkin's disease.

There has been much enthusiasm until recently for the BEACOPP (bleomycin, etoposide, doxorubicin, cyclophosphamide, vincristine, procarbazine, and prednisone) and escalated BEACOPP regimens developed by the German group. In an early report [157], moderate dose escalation over the standard BEACOPP regimen was performed with granulocyte colony-stimulating factor (G-CSF) support, and maximal doses of three component drugs (doxorubicin, etoposide, cyclophosphamide) were given with acceptable hematological toxicity. The intended doses of doxorubicin, etoposide, and cyclophosphamide were substantially escalated from 25 to 35, from 650 to 1200, and from 100 to 200 mg/m^2, respectively. Of 60 patients enrolled, 56 (93%) achieved complete remission, with an overall survival and freedom from progression at 32 months of 91% and 90%, respectively. Another report gave the results of a randomized comparison of standard BEACOPP with escalated BEACOPP and COPP–ABVD, involving 505 evaluable patients with advanced-stage (IIB, IIIA with risk factors, IIIB, and IV) disease. Four courses of COPP–ABVD were compared with eight courses of baseline BEACOPP without G-CSF and with eight courses of escalated BEACOPP with G-CSF, all followed by radiotherapy to bulky sites (30 Gy) and to areas of residual disease (40 Gy) [157]. The interim analysis at 23 months showed a significant inferiority of the COPP-ABVD regimen in terms of progression rate and freedom from progression compared with the pooled results of both BEACOPP schemes. The 24-month freedom from progression rate was 75% for COPP/ABVD and 84% for BEACOPP pooled ($p = 0.034$). There were insufficient data to compare the two BEACOPP variants. The acute toxicity of COPP/ABVD and baseline BEACOPP was similar, whereas escalated BEACOPP showed increased but manageable hematological toxicity. Therefore, recruitment in the COPP/ABVD arm was discontinued. In a further interim analysis of 689 patients (COPP/ABVD 235 patients, baseline BEACOPP 241, escalated BEACOPP 213), escalated BEACOPP showed better freedom from progression at 2 years than baseline BEACOPP (89% vs. 81%). As yet, no significant differences in survival have emerged, however. Of concern is the higher number of secondary acute leukemias observed with escalated BEACOPP (4 vs. 1) whereas secondary non-Hodgkin's lymphomas (three cases) occurred only in the baseline BEACOPP group. Nevertheless, the authors concluded that results with escalated BEACOPP in advanced-stage Hodgkin's lymphoma challenged ABVD as the standard of care for this population [213]. The same study group tested BEACOPP without etoposide (BACOPP) in elderly patients (age 60–75 years) with early-stage disease with unfavorable characteristics, or advanced-stage. The regimen was effective in this population but there was an unacceptably high toxic death rate of 11% [214]. In an important presentation, Gianni et al. [215] demonstrated comparable 3-year overall survival with ABVD or BEACOPP first-line therapy for advanced-stage patients plus autologous transplant as salvage for relapse. Freedom from progression rate at 3 years was 16% higher in the BEACOPP group, but the freedom from second progression rates were similar, as were overall survival rates. The authors concluded that 71% of the ABVD-treated patients were cured by initial therapy and would have been overtreated if they had received

BEACOPP instead. BEACOPP was associated with a fourfold higher toxic death rate compared with ABVD.

In the HD12 trial, the German Hodgkin's Study Group attempted to determine whether eight cycles of escalated BEACOPP followed by irradiation might represent overtreatment. Advanced-stage Hodgkin's lymphoma patients were randomized to receive four or eight cycles of escalated BEACOPP followed by four cycles of baseline BEACOPP. A second randomization assigned patients to receive radiotherapy as described in the previous trial or no radiotherapy. The study demonstrates that substantial reduction in irradiation dose after BEACOPP chemotherapy is warranted [216].

Among numerous newer multidrug regimens in which etoposide was substituted for vincristine and epidoxorubicin for doxorubicin, it is worth mentioning VEBEP (etoposide, epidoxorubicin, bleomycin, cyclophosphamide, and prednisone). The Milan group reported their results with this regimen given every 21 days without growth factor support and followed by radiation therapy (30–36 Gy) to pretreatment-involved nodal sites. In 73 patients with stages IIB or III–IV or relapsing after radiotherapy, complete remission was achieved in 94% of cases with freedom from progression and overall survival at 6 years of 78% and 82%, respectively. Hematological toxicity was acceptable, with 86% grade IV neutropenia, which resolved in the majority of cases by the next treatment cycle. The majority of male patients developed gonadal damage, reversible in half of them. No secondary leukemia or myelodysplasia had been detected at the time of publication [217]. Overall (Table 43.9), these regimens as well as others induce complete remission in up to 99% of cases with freedom from progression at 5 years of about 80%. None of these regimens are clearly superior to ABVD.

It has been demonstrated that bleomycin and vincristine doses can be reduced or omitted from the BEACOPP regimen without appreciable loss of efficacy, which raises the question how important these agents are in the treatment of Hodgkin lymphoma [218]. On the other hand, it has been demonstrated that dacarbazine cannot be omitted from the ABVD regimen without loss of significant activity [200].

Table 43.9 Five-year results of chemotherapeutic regimens in advanced Hodgkin's disease

Regimen (ref)	CR (%)	OS (%)	FFP (%)
BEACOPP intensified [213]	95	NR	84
MVC [219] (88% of patients had B symptoms)	90	NR	79
VEBEP [217]	94	82	78

CR complete remission, *OS* overall survival, *FFP* freedom from progression
Whatever treatment is to initiated for a given patient, it is important to begin treatment as soon after diagnosis as possible [SSSS] because delay may lead to inferior results

A simple regimen of mitoxantrone, 8 mg/m^2 given I.V. daily on days 1–3 plus vinblastine, 8 mg/m^2 I.V. on days 1 and 22, and lomustine orally 100 mg/m^2 on day 1 (MVC) has yielded results similar to more complicated regimens [219] and deserves further study.

Newer drugs in development such as brentuximab vedotin are under investigation in combination with other agents for the treatment of newly diagnosed patients with advanced disease [220]. Such combinations may ultimately render present-day regimens obsolete. New agents in development are discussed below [221].

High-Dose Consolidation with Stem Cell Support

A number of nonrandomized trials have tested high-dose consolidation after achievement of complete remission with standard regimens in patients with particularly adverse prognostic presentations. Up to now there has been no evidence for the usefulness of such an approach in any subset of patients. As a matter of fact, up to 40% of relapsed patients are cured with high-dose chemotherapy as salvage treatment (see discussion that follows). Thus, giving high-dose consolidation therapy may be overtreatment.

Carella et al. studied one course of CBV (cyclophosphamide, BCNU, etoposide) or BEAM (BCNU, etoposide, cytarabine, melphalan) followed by peripheral stem cell reinfusion in 22 patients with advanced-stage disease in first remission after MOPP-ABVD. Selection criteria consisted of bulky disease, age over 40 years, high serum lactate dehydrogenase (LDH) level, and anemia at onset. After a median follow-up time of 83 months, overall and RFS were 80% and 77%, respectively [222].

Delain et al. [223] treated 26 high-risk patients with an estimated probability of cure less than 40% (stage IV and at least two of the following factors: B symptoms, bulky mediastinal disease, two or more extranodal sites, bone marrow involvement, inguinal node involvement, high LDH serum level, or low hematocrit) with high-dose chemotherapy and peripheral blood stem cell support (PBSCT) as consolidation of first-line chemotherapy. At the time of transplantation, 19 patients were in complete remission and seven in good partial remission after MOPP-ABVD or similar regimens. Actuarial 5-year overall survival, freedom from progression, and event-free survival were 69%, 79%, and 58%, respectively. Only normal serum LDH level was found statistically significant as a prognostic indicator in univariate analysis. Of note, procedure-related mortality in the first 90 days after engraftment was 7%. Better results were obtained by Nademanee in 20 high-risk patients according to the same criteria, who were given high-dose therapy and total-body irradiation after complete remission induction. All patients were alive and disease-free at

43 months [224]. Zinzani et al. [131] utilized autologous stem cell transplantation after high-dose chemotherapy in advanced-stage patients who were PET scan-positive after two cycles of ABVD and compared results with patients who were PET scan-negative after two cycles of ABVD. The 2-year progression-free survival was 76% for the patients who were PET+ during ABVD treatment and 81% for those who were PET-negative. The authors concluded that the intense chemotherapy with transplantation allowed the poor responders to ABVD to have a similar outcome as the ABVD complete responders. Again, it must be stressed that all these results come from small nonrandomized heterogeneous series and must be evaluated with caution.

Role of Consolidation Radiotherapy in Advanced Stage

Soon after MOPP was introduced, it became clear that the majority of chemotherapy relapses occurred at sites of initial disease, particularly nodal sites and sites of bulky tumor, and that addition of radiotherapy markedly reduced the frequency of these recurrences, thus increasing the rates of both complete remission and disease-free survival [225, 226]. Because of these observations, adjuvant irradiation was widely adopted. However, with growing awareness of the second tumor risk associated with irradiation, both radiotherapists and chemotherapists have reservations about this approach [227, 228].

There is general agreement on the need for combined-modality treatment in patients with mediastinal bulky disease [227–229]. Disease-free survival of 80–90% is observed after combined-modality treatment, which is nearly twice that reported after MOPP or radiation alone. Some partial remissions obtained with chemotherapy can be converted to complete remissions with radiotherapy, but such patients do not show an increased cure rate according to randomized trials [229, 230]. So-called risk-adapted therapy which consists of intensifying chemotherapy or adding radiotherapy to standard chemotherapy if a PET scan obtained after two courses of the latter shows residual disease is under investigation in a number of studies but, to date, results are controversial [231, 232].

The issue of whether irradiation has a role as consolidation therapy after complete remission is achieved is still a matter of debate. Groups at Yale, Duke, and Memorial Sloan-Kettering strongly support low-dose adjuvant irradiation (18–30 Gy) to previously involved sites for patients in remission after chemotherapy. In a cohort of 184 Yale patients with either newly diagnosed stage IIIB or IV disease or recurrent disease after irradiation alone, overall survival was 54% at 15 years [233]. However, an unacceptable incidence of second neoplasms was observed in those treated with combined-modality therapy for disease recurrence after radiation alone (41% at 20 years vs. 12% in the newly diagnosed patients) [181]. In a similar analysis at Memorial Sloan-Kettering, the actuarial 10-year overall survival and progression-free survival were 74% and 70%, respectively [234]. Second-malignancy incidence was only 4%, but the median duration of observation was too short to properly address that issue. A German investigation showed that while 20 Gy is sufficient to control initial sites of nonbulky disease or uninvolved sites following two double cycles of COPP/ABVD relapse patterns indicate that patients destined to relapse need more systemic rather than local therapy [235]. In a SWOG study, 278 patients in complete remission following a MOPP–ABVD-derived regimen were randomized to receive low-dose irradiation (10–20 Gy) to previously involved sites or no further treatment. The 5-year remission duration estimated at 79% for patients who received radiation therapy was not significantly different from the 68% observed in those who did not. Although low-dose irradiation improved the 5-year remission duration in the subgroups with nodular sclerosis (82% vs. 60%, $p = 0.002$) or bulky disease (75% vs. 57%, $p = 0.05$), overall 5-year survival was not improved in any subgroup [236]. The GELA group [237] published the results of a randomized comparison between two cycles of chemotherapy and nodal radiotherapy consolidation for patients with stage IIIB-IV Hodgkin's disease in complete remission or good partial remission after six cycles of MOPP-ABV hybrid or ABVPP (doxorubicin, bleomycin, vinblastine, procarbazine, prednisone). After induction therapy, 418 patients were evaluable for consolidation treatment comparisons. After a median follow-up period of 48 months, the 5-year freedom from progression did not differ between chemotherapy (79%), and combined modality (74%). After MOPP-ABV, 5-year overall survival was similar for chemotherapy (85%) and combined modality (88%); after ABVPP the 5-year survival was 94% for chemotherapy and 78% for combined-modality therapy. In conclusion, these results do not support the use of consolidation with radiotherapy instead of two further courses of chemotherapy after doxorubicin-induced complete remission for patients with advanced Hodgkin's disease.

In 1998 [203], the International Database on Hodgkin's Disease Overview Study Group published a meta-analysis of 1740 patients from 14 controlled adjuvant irradiation clinical trials. In studies comparing the addition of radiation to chemotherapy versus the same chemotherapy alone, tumor control at 10 years was significantly improved by 11%, but overall survival was similar. Trials in which the addition of radiation to chemotherapy was compared with the addition of further chemotherapy showed that there was no difference in tumor control in the two groups if an appropriate number of drug cycles were administered. However, overall survival was 8% better in the chemotherapy alone group because of fewer late treatment-related deaths. Adjuvant radiotherapy was thus recommended only for a few specific indications

such as bulky mediastinal disease. Thus, according to "evidence-based-medicine" criteria, there is no clear indication for consolidation therapy after complete remission in advanced stages of Hodgkin's disease.

Salvage Therapy

The vast majority of relapsing patients will require some form of systemic therapy. The issues involved in salvage treatment of Hodgkin's disease revolve around such concepts as non-cross-resistant chemotherapy, multidrug resistance, and response to escalated doses of chemotherapeutic agents [238, 239].

Prognostic Factors and Choice of Treatment

Salvage treatment for Hodgkin's lymphoma is required for essentially four subsets of patients with different prognostic implications (1) patients relapsing after radiotherapy alone; (2) patients relapsing after more or (3) less than 12 months from the achievement of complete remission with first-line chemotherapy; and (4) chemoresistant induction failure patients. At present, the widespread use of high-dose chemotherapy with stem cell support in virtually all patients relapsing after chemotherapy has eliminated the distinction between categories (2) and (3) (see discussion that follows). As a matter of fact, the number of cases in the first group is progressively decreasing, as only a few patients are now given front-line radiotherapy alone (see earlier text).

In the past, duration of first complete remission has been considered the main prognostic factor predicting second chance of cure. In fact, a number of trials demonstrated prolonged disease-free survival by retreatment with the same regimen that resulted in a prolonged initial complete response. Of 32 patients relapsing after primary chemotherapy at the NCI, 59% achieved a second complete remission when retreated with MOPP [240]. However, only 29% of patients whose initial remission was less than 1 year achieved a second complete remission, compared with 93% of patients whose initial complete remission was >1 year. The duration of second remission was also longer in patients whose initial complete remission exceeded 1 year than in those whose initial remission was less than 12 months. Similar results were also reported by the Milan group [241, 242], as well as by other investigators.

Today, the duration of first complete remission represents only one of the prognostic variables considered when second-line treatment is planned. A number of other factors, such as extension of disease at relapse, presence or absence of B symptoms, performance status, and response rate to induction therapy preceding high-dose consolidation, have demonstrated their prognostic significance in this setting [243].

Patients over the age of 60 are at increased risk for lung toxicity of bleomycin. Several recent studies have suggested that, while ABVD is currently preferred over other regimens for treatment of elderly patients, no more than two courses should be given with or without radiation therapy [244–247]. In the future ABVD may be replaced by brentuximab vedotin alone [248] or in combination with dacarbazine [249] or other agents for the initial treatment of elderly patients.

Relapse

Initial relapses of Hodgkin lymphoma tend to occur predominantly in the first 2–4 years after completion of treatment. Late relapses (beyond 5 years) are uncommon and account for 5–15% of patients in reported series [240, 250–252]. Relapses decades after initial diagnosis have occasionally been reported [253, 254].

In patients treated with inadequate radiation therapy, new manifestations of lymphoma are usually observed in the adjacent lymph node chain(s). In these cases, new disease manifestations in noncontiguous untreated areas are relatively rare. True recurrence (relapse within an irradiated field) is rare. Marginal recurrence, relapse at or immediately adjacent to the margins of a previous radiation field, is more frequent and may occur in 15–25% of cases treated with radiotherapy alone [252]. A trend toward recurrence in nodal areas and particularly in initial and contiguous sites can also be seen after complete remission in Hodgkin lymphoma treated with chemotherapy. In fact, relapses in previously involved sites occur in about 70% of relapsed cases after chemotherapy alone and are directly related to the volume of tumor mass prior to therapy.

Relapse after Radiation Therapy

As previously noted, 20–40% of patients with stages I and II Hodgkin lymphoma who are treated with primary radiation therapy will eventually relapse within 3 years. In a few instances, radiation therapy alone has been successfully used to salvage patients when the relapse occurred in a single lymph node chain more than 5 years after completion of the initial radiotherapy. In all other cases, the survival of patients treated with chemotherapy after radiation relapse is at least equal to that of advanced-stage patients initially treated with the same chemotherapy combination. Indeed, an apparent survival advantage of patients with radiation relapses over the primary treatment group has been attributed to a more favorable patient mix, with generally more limited extension of disease. Overall and disease-free survival range from 60% to 80% [250, 255].

Even in this case, stage at relapse is an important prognostic variable. At Stanford the 10-year relapse-free survival was 88%, 58%, and 34%, respectively, for those in stage IA, in stage IIA or IIIA, and in stage IV or with B symptoms at the time of relapse. As far as the choice of chemotherapy regimen is concerned, the same considerations outlined for first-line chemotherapy are of value. From the available evidence, ABVD exhibits the same superiority over other regimens for post-radiation recurrence that it does in initial treatment of advanced disease [149, 151].

Relapse After Chemotherapy

The treatment of choice for patients relapsing after chemotherapy has been historically represented by non-cross-resistant regimens at conventional doses. In the past, relapses after MOPP could be safely treated with ABVD with a second chance of cure as high as 40% [241]. However, the vast majority of patients now receive front-line treatment with ABVD or some variant of it. Thus, a number of new regimens have been devised that induce complete remission in no more than 50% of all patients, with 50–70% of the responders eventually relapsing again. Such patients have a probability of survival at 5 years after relapse of only 15–30% [256–258].

Somewhat better results come from regimens containing intermediate- or high-dose ifosfamide combined with etoposide or new active drugs such as vinorelbine [259–261]. In small series, up to 70% of patients are disease-free after 2 years [259], and of note, there is no difference in outcome between relapsed and primary refractory cases. MVC mitoxantrone 8 mg/m^2 on days 1–3, vinblastine 8 mg/m^2 days 1 and 22, and lomustine 100 mg/m^2 day1 is a new regimen given every 6–8 weeks for four cycles. A 91% CR rate with a median duration of response of 11 months and a median overall survival rate of 34% was reported in 45 relapsed patients most of whom received ABVD as initial treatment [219].

Several new drugs in early clinical development have shown substantial clinical activity against Hodgkin lymphoma and may ultimately revolutionize its treatment. Brentuximab vedotin is an anti-CD30 monoclonal antibody coupled to a tubulin inhibitor, monomethyl auristatin. The antibody binds to CD30 on Reed–Sternberg cells. The entire compound is then internalized, the linker is cleaved and monomethyl auristatin is released in the cytoplasm and causes apoptosis [262]. The first phase I study in patients with relapsed or refractory CD30+ lymphoma (Hodgkin lymphoma and anaplastic large cell lymphoma) tumor regression was observed in 86% of evaluable patients [263]. In a subsequent phase II trial of 102 relapsed or refractory Hodgkin lymphoma patients who failed an autologous stem cell transplant and then received brentuximab vedotin, 34 patients achieved a complete remission and 16 of those remained progression-free after a median follow-up of >53 months [264]. In another phase II study in relapsed or refractory patients who had received and failed salvage therapy but had not had a stem cell transplant, 9 of 30 patients had a complete response by PET scan after brentuximab vedotin alone [265]. A meta-analysis was performed on 903 Hodgkin lymphoma patients reported in the literature up to July 1, 2015, who received multiple treatments for relapsed or refractory lymphoma (including 529 patients who had received high-dose chemotherapy and an autologous stem cell transplant) and were subsequently treated with brentuximab vedotin [266]. The overall response rate was 63% and the complete response rate was 32%. Other recent studies [267–270] have given equally impressive results. The toxicity of brentuximab vedotin in these studies has been primarily grade 1–2 hematologic, fatigue, nausea and peripheral sensory neuropathy, which resolves completely or partially after discontinuation of treatment. In general, the drug has been well tolerated although approximately one-third of patients had treatment delayed due to toxicity at some point. Rarely severe dermatologic reactions, acute pancreatitis, or progressive multifocal leukoencephalopathy may occur. The usual dose and schedule is 1.6–1.8 mg/kg intravenously every 3 weeks for eight to ten cycles. This agent will almost certainly be studied in combination with other agents for newly diagnosed and refractory or relapsed patients in the near future.

Nivolumab, a PD-1 blocking antibody was recently reported to have major activity in relapsed or refractory Hodgkin lymphoma [271]. Most of the patients in this small phase II study had failed an autologous stem cell transplant and/or brentuximab vedotin. Yet 17% had a complete response and 70% had a partial response to nivolumab, 3 mg/kg I.V every 2 weeks on an open ended schedule. Toxicity of any grade occurred in 78% and grade 3 toxicities were observed in 22% of patients. These impressive results will insure that this agent will be fully evaluated as a treatment for Hodgkin lymphoma. Pembrolizumab [272], a humanized monoclonal anti-PD-1 blocker is also under investigation in relapsed and refractory Hodgkin lymphoma.

Bendamustine [273, 274] has also shown significant activity in previously heavily treated relapsed or refractory patients with Hodgkin lymphoma, even after failure of brentuximab vedotin [275].

90Y–daclizumab, a radiolabeled anti-CD25 monoclonal antibody given I.V. produced 14 complete responses in 46 evaluable relapsed patients with Hodgkin lymphoma in a recent phase II study. Unfortunately, six patients developed a myelodysplastic syndrome after treatment [276]. Whether this was due to previous treatments or the antibody is not clear. Further studies are necessary. Other new agents in early preclinical or clinical development that show activity against Hodgkin lymphoma include auranofin [277], AFM13 [278] and ixazomib [279].

For the first time in decades we have new agents with substantial activity against Hodgkin lymphoma. Clinical investigators throughout the world will be occupied for years to come determining the best ways to capitalize on this embarrassment of riches.

Stem Cell Transplantation

High-dose therapy with autologous stem cell rescue (Tables 43.10 and 43.11) is applicable to a large proportion of patients with relapsed or refractory Hodgkin lymphoma. On examining the literature (Table 43.10), a 5-year freedom from progression of approximately 40–50% emerges despite the heterogeneity of case series in terms of the number of patients treated, induction and conditioning regimens, and eligibility criteria [167, 280–286].

Recent trials (Tables 43.10 and 43.11) suggest the possibility of cure by high-dose therapy even in patients with primary refractory disease, defined as those never achieving complete remission with first-line chemotherapy. Lazarus et al. [289] reported on 122 chemotherapy-resistant patients who obtained a complete response rate of 50% with high-dose therapy and a 38% 3-year overall survival. Similar data

Table 43.10 High-dose therapy with stem cell rescue for patients in first relapse

Author (ref)	*N*	Selection criteria	Conditioning regimen	FFP (%)	FU (years)
Chopra [280]	52	Relapse within 1 year	BEAM	47	5
		At least two previous regimens			
Nademanee [224]	43	Relapse within 1 year	BCNU/VP16/CTX	40	3
		Non-CR after salvage therapy	TBI/VP16/CTX		
Bierman [281]	58	Relapse after chemotherapy	CBV	40	5
Reece [282]	58	Relapse after chemotherapy	CBV ± CDDP	61	5
Yuen [255]	47	Relapse after chemotherapy	BCNU/VP16/CTX	56/50	4
		1 year, more or less	TBI/VP16/CTX		
Wheeler [283]	42	Relapse after CR	CBV	44	4
[a]Sweethenham [284]	139	Relapse after CR	BEAM/CBV/others ± TBI	44.7	5
[b]Brice [287]	220	Relapse after CR	CBV/BEAM/BEAC/others	71	5

BEAM BCNU, etoposide, cytarabine, melphalan, *CTX* cyclophosphamide, *CBV* cyclophosphamide, BCNU, etoposide, *TBI* total-body irradiation, *CDDP* cisplatin, *BEAC BCNU*, etoposide, cytarabine, cyclophosphamide, *CR* complete remission, *FFP* freedom from progression, *FU* follow-up period
[a]Data from the International Bone Marrow Transplantation Registry
[b]Data from the Societé Française de Graffe de Moelle

Table 43.11 High-dose therapy with stem cell rescue for patients with primary refractory disease

Author (ref)	*N*	Selection criteria	Conditioning regimen	FFP (%)	FU (years)
Gianni [288]	16	Non-CR with first-line MOPP/ABVD	SCT + TBI or IFRT	31	6
Chopra [280]	46	Non-CR with MOPP, MOPP/ABVD	BEAM	33	5
Reece [285]	30	Histologically confirmed persistent disease	CBV ± CDDP	42	4
Horning [243]	29	PR or PD during CT: PD within 4 weeks from chemotherapy completion	TBI/VP16/CTX BCU(CCNU)/VP16/CTX	50	4
Lazarus [289]	129	Histologically confirmed persistent disease	High-dose therapy ± TBI	38	3
Musso [290]	122	Relapsed or refractory lymphoma	Fotemustine substituted for BCNU in BEAM	74	2
Sweethenham [284]	290	SD or PD after first or second line	BEAM/CBV/others ± TBI	30	5

SCT high-dose sequential therapy, CTX → methotrexate → VP16 → TBI/melphalan, *IFRT* involved-field radiotherapy, *CR* complete remission, *SD* stable disease, *PD* progressive disease, *FFP* freedom from progression, *FU* follow-up period

were provided by a German group [291], which reported a 5-year overall survival of 19% among 67 patients with primary refractory disease. Of note, overall survival was 53% for the 25 patients who actually could receive high-dose consolidation. This somewhat lower probability of cure as compared with the International Transplant Registry data is considered to result from different data methodology in a single center than that employed for the multicenter database. The GELA/SFGM group studied an especially unfavorable group of patients with relapsed or refractory Hodgkin's disease and demonstrated the feasibility of tandem administration of high-dose chemotherapy [287]. Of 43 patients, 32 (74%) completed the treatment. Platelet recovery after the second transplant (TBI or busulfan-containing preparative regimen) was delayed. There were two veno-occlusive disease episodes, one fatal, and one case of hemorrhagic cystitis, but no grade IV toxicity with a reduced dose of busulfan. The 2-year survival for patients receiving the two procedures was 74%, and that for the whole series was 65%.

The mini-BEAM regimen is certainly efficacious, but stem cell collection is impaired after its use [288, 292]. Stem cell collection may be improved by plerixafor [293]. The Cologne group demonstrated the feasibility of giving three or four courses of dexaBEAM prior to high-dose BEAM with adequate stem cell collection, which suggests the need to achieve as maximal a response as possible with conventional induction therapy prior to high-dose therapy [291]. Other regimens containing etoposide, platinum compounds, cytarabine, ifosfamide, or vinorelbine are also frequently used and combine antilymphoma activity with stem cell-mobilizing potential [294].

Further improvement of outcome for patients with relapsed Hodgkin lymphoma should focus first of all on increasing the complete remission rate with induction therapy, which may be achieved by increasing the dose intensity of effective regimens, or by including new active drugs, such as gemcitabine, vinorelbine [295], or mitoxantrone [219] in induction regimens. Second, the concomitant use of immunologically targeted strategies such as immunotoxins or monoclonal antibodies should be explored in this setting with the aim of reducing minimal residual disease [296, 297].

Some have advocated tandem autologous stem cell transplantation for very poor-risk patients [298] but this approach may not be necessary in light of new antilymphoma agents now available.

The question that still is not answered is whether or not autologous stem cell transplantation after high-dose chemotherapy ± irradiation is superior to other treatments for relapsed or refractory Hodgkin lymphoma patients. A review of 154 patients transplanted at Stanford from 1988 to 2002 and survived ≥2 years revealed 54 deaths after a median follow-up of approximately 10 years (34 from Hodgkin lymphoma, 13 from second malignancies and seven from other causes). The number of deaths from Hodgkin lymphoma is not less than one would expect from relapsed or refractory Hodgkin lymphoma patients treated otherwise [299], and one would expect the number of deaths from second malignancies to increase as follow-up continues.

There are three trials in the literature in which relapsed or refractory Hodgkin lymphoma patients were randomized to receive an autologous stem cell transplant-based treatment or a non-transplant treatment. Two meta-analyses (by the same authors) of these trials showed no overall significant survival benefit for transplanted patients [300, 301].

Allogeneic stem cell transplantation has been studied in relapsed and refractory Hodgkin lymphoma, including with reduced intensity conditioning [302] and with umbilical cord stem cells [303, 304]. At least one meta-analysis found no apparent survival plateau in these studies [305]. Furthermore, there is a high incidence of post-transplant lymphoproliferative disease in some of these studies [304], which impairs survival [306]. Currently, less than half of patients with relapsed or refractory Hodgkin lymphoma are cured with standard salvage chemoradiotherapy followed by high-dose therapy and autologous stem cell transplantation [307]. It is likely that stem cell transplantation for Hodgkin lymphoma will suffer the same fate it did as treatment for chronic myeloid leukemia as new agents for the lymphoma currently under study are perfected.

Treatment Sequelae

Unfortunately, recent improvement in the cure rate of Hodgkin's disease patients has been partially offset by treatment-related mortality and morbidity. In the Stanford experience [308], 15 years after diagnosis the mortality rate from causes other than Hodgkin's disease is greater than that from Hodgkin's disease itself (Table 43.12). Because the median age of patients 15 years after treatment was only 44 years, mortality from causes other than Hodgkin's disease is a serious problem. Few additional disease-related deaths occur beyond 15 years, although late treatment-related deaths continue to occur. Quality of life in some long-term survivors may be significantly impaired, according to a "fatigue score" [309].

Cardiac Complications

Cardiovascular complications of mantle irradiation and anthracycline chemotherapy are the second most frequent cause of treatment-related mortality in Hodgkin's lymphoma patients. Valvular heart disease incidence is directly related to mediastinal radiation dose, and with modern techniques that employ 30Gy or less the 30-year risk is increased by only about 1.4% [310].

Table 43.12 Causes of death among 2498 patients treated at Stanford University [308]

Cause of death	*N*	Percent
Hodgkin's disease	333	44
Second neoplasms	160	21
Cardiovascular	117	16
Pulmonary	50	7
Infection	31	4
Accident	14	2
Hematologic	9	1
Gastrointestinal	4	1
Other	14	2
Unknown	22	3

In a Dutch study of 2524 survivors of Hodgkin lymphoma [311] 1713 cardiovascular events were identified in 797 patients. After 35 years or more patients had a four- to six-fold increased incidence of coronary artery disease or congestive heart failure compared with the general population. The highest relative risks were observed in patients treated before the age of 25 years, and half of patients with cardiovascular disease developed multiple events. Mediastinal radiation increased the risk for coronary artery disease and anthracyclines increased the risk for valvular disease. In another large retrospective study of 1919 patients diagnosed at a median age of 30 years, [312]1238 cardiac events were recorded in 703 patients after a median follow-up of 9 years: 19% were ischemic heart disease, 12% were congestive failure, 16% were arrhythmias and 11% were valvular disease. Older patients, Black patients, and men, especially those who presented with advanced-stage lymphoma appear to be at highest risk of cardiovascular death after treatment for Hodgkin lymphoma [313, 314]. Recent evidence suggests that vigorous exercise after lymphoma treatment may favorably modify the incidence of treatment-related cardiovascular events [315–318]. Coronary computed tomography angiography has been recommended as a follow-up procedure especially for patients treated with combined-modality therapy at a young age [319].

Radiation-related pericarditis occurs in 25–30% of patients treated with radiation therapy. It is usually transient and self-limiting, but constrictive pericarditis requiring surgery with or without tamponade may occur [197] as late as 10 years after mediastinal irradiation [320]. The early postoperative outcome of cardiac surgery for radiation-related cardiac damage is generally like that of the population as a whole [321].

Pulmonary Complications

Pulmonary morbidity and mortality are related both to mantle field irradiation and to bleomycin [322]. Major risk factors include age over 40 years [323] cumulative dose of bleomycin as high as 300–400 U/m^2, concomitant mediastinal irradiation, and oxygen therapy [325]. In addition, the administration of both bleomycin and gemcitabine together leads to a substantial increase in pulmonary toxicity above that expected with bleomycin alone [324].

Bleomycin-induced mortality after ABVD occurs in approximately 4% of patients [325]. The primary lung lesion is macrophage-rich fibrosis. Careful monitoring of respiratory function during treatment may be helpful in decreasing such a risk in that bleomycin can be discontinued if impaired pulmonary function is detected early [325] and doing so has no effect on complete remission rate, PFS or overall survival [326]. This observation raises the question whether bleomycin adds any therapeutic activity to the ABVD regimen at all (discussed above). Early radiation pneumonitis and to some extent bleomycin pneumonitis may respond to corticosteroid administration, but late radiation pulmonary fibrosis is untreatable [323]. PET scanning may be more helpful than CT scanning in following the evolution of bleomycin pulmonary toxicity after treatment [322]. Patients with radiation or bleomycin lung toxicity usually have impaired exercise tolerance, especially if decreased DLCO and decreased total lung capacity are demonstrated [327]. A number of agents have been shown in rodents to prevent or lessen bleomycin pulmonary activity [328–330] but none have entered clinical trial yet.

Second Neoplasms

The mechanisms involved in the development of second malignancies in Hodgkin lymphoma are likely based on the damage and repair at a gene level caused by treatment. Alterations in cell cycle, repair, detoxification, and stress response pathways are likely involved [331]. Interestingly, Hodgkin's lymphoma patients with a family history of cancer are more likely to develop a second malignancy after treatment and the risk increases directly with the number of first-degree relatives with cancer [332].

When MOPP was the standard chemotherapy regimen, acute myeloid leukemia and myelodysplasia were the first second malignancies to be observed. They occurred primarily from months to several years after completion of chemotherapy, primarily due to nitrogen mustard and procarbazine. The occurrence of acute leukemia after ABVD or Stanford V treatment is extremely rare [333, 334]. Radiation therapy does not seem to play a role in acute leukemia etiology [333]. The overall incidence of acute myeloid leukemia and myelodysplasia within 10 years from initial treatment for Hodgkin lymphoma ranges in most series from 1% to 4% or less. The highest incidence of this complication occurs in patients treated with extensive radiation therapy plus MOPP or MOPP-like chemotherapy, in patients older than 40 years at the start of chemotherapy, and in those subjected to staging

laparotomy with splenectomy. Furthermore, it seems that patients who initially undergo radiation therapy and subsequently receive salvage chemotherapy represent the subgroup at highest risk for developing leukemia or myelodysplasia. This is a refractory hematologic neoplasm and virtually no patients survive this complication [335–337]. The excess risk for AML is highest in patients diagnosed with Hodgkin's lymphoma at age 35 years or older, and has declined after 1984, when MOPP began to be phased out [338]. In summary, the incidence of AML and myelodysplasia in patients treated for Hodgkin lymphoma is directly related to the cumulative dose of alkylating agents [339] or BCNU [340] received.

Typically, treatment-related leukemia is characterized by a latency period of months to 5 years or more, a preceding myelodysplastic phase, trilineage bone marrow dysplasia, and abnormalities of chromosome 5 and/or 7 [337]. Topoisomerase II inhibitors, primarily epipodophyllotoxins but also anthracyclines when given in combination with alkylating agents, have also been implicated in the development of a clinically and cytogenetically distinct form of secondary acute myeloid leukemia in both adult and pediatric patients. In contrast to alkylating agent-related leukemia, this form of therapy-related leukemia lacks a preceding myelodysplastic phase and is characterized by a shorter latency period, myelomonocytic or monocytic morphology, and balanced chromosome translocations involving bands 11q23 or 21q22. The cumulative dose of epipodophyllotoxins as well as dose-dense administration are implicated in the induction of this type of secondary leukemia [341].

Hematological malignancies have been studied also in survivors after high-dose therapy with stem cell support [342, 343]. It must be emphasized that acute myeloid leukemia and myelodysplasia often result from chemotherapy and/or radiation therapy given for the primary malignancy prior to transplantation rather than from high-dose chemotherapy administered as a transplant preparative regimen. As a matter of fact, the latency period from first-line therapy and development of leukemia is 4–6 years, whereas it is substantially less from the high-dose chemotherapy–PBSCT time (median 2.7 years) [342, 343]. In a report from the University of Chicago Medical Center concerning 641 patients, of whom seven (1.1%) developed therapy-related acute leukemia [341]. The disease-specific incidence of therapy-related leukemia was 6.3% of patients treated for Hodgkin lymphoma. The median latency periods from the time of initial treatment and from that of high-dose therapy were 5.5 and 1.5 years, respectively [342, 343]. Thus, it is more likely that the development of secondary leukemia resulted from pretransplant chemotherapy, which implies that early detection of therapy-related cytogenetic abnormalities may be useful to exclude patients from high-dose chemotherapy–PBSCT programs [344].

A small number of patients treated with intensive combined-modality therapy have developed aggressive non-Hodgkin's lymphoma shortly after completing treatment. This transformation is especially common in patients with nodular lymphocyte-predominant Hodgkin lymphoma for whom a >10% conversion to diffuse large B-cell lymphoma has been reported [345]. In a series of 1391 Hodgkin's disease patients treated with various approaches from 1972 to 1996, the possible role of splenectomy in the development of non-Hodgkin's lymphoma was investigated. After a median follow-up period of 84 months, a total of 20 cases of non-Hodgkin's lymphoma were observed, with a cumulative risk of 0.8% at 5 years and 3.5% at 20 years. Splenectomy or splenic irradiation and age over 40 years were associated with relative risks of 5.69% and 3.05% respectively, for unknown reasons.

The major second malignancy problem in Hodgkin lymphoma patients is the development of solid tumors most often within irradiation fields years to decades after completion of treatment. That risk continues into the fourth decade and probably beyond [346]. These tumors are mostly due to radiation therapy, but some data suggest that MOPP chemotherapy may lead to an elevated risk of lung cancer in patients with Hodgkin's lymphoma [347]. The risk is no greater after conventional therapy followed by autologous stem cell transplantation than after conventional therapy alone.

The literature has focused mainly on secondary acute myeloid leukemia, myelodysplasia, and breast cancer as important second neoplasms after treatment for Hodgkin lymphoma. However, lung cancer [348–351], sarcomas [351], head and neck cancer (especially salivary gland tumors) [352, 353], and gastric cancer [354] occur with increased frequency as well. The median latency period is relatively short for acute myeloid leukemia and myelodysplasia, but it may be as long as 18 years or more for lung cancer [351], which is primarily adenocarcinoma. High doses of mediastinal irradiation and smoking increase the risk of lung cancer after lymphoma treatment. Lower doses of radiation may [355] or may not [356] reduce the risk of second solid tumors.

The excess risk of breast carcinoma is almost completely restricted to women who received radiation therapy before the age of 30 [136]. Those treated in the second decade and the first half of the third decade of life are subject to the maximum cumulative risk [358], with shoulders of the risk peak extending into the second half of both the first and third decades. The risk curve is consistent with the current understanding of breast carcinogenesis. In fact, the first breast carcinomas appear at the end of the first decade after irradiation [358] and continue to appear in women observed for the longest period (three decades). In one series, actuarial calculation observed an overall 19% incidence of breast cancer 30 years after irradiation and 26% for those treated before age 21 [358]. A recent large population-based study puts the

incidence at 6.5% [357]. That study found a decline in the use of radiation therapy for the treatment of Hodgkin lymphoma from 1973–2011 and a consequent fall in the incidence of secondary breast cancer during that period. These second tumors appear within or at the edge of irradiation fields [359], and shorter duration of intact ovarian function after irradiation was associated with a significant reduction in breast cancer risk [358]. Systematic follow-up of women who received radiation treatment for Hodgkin's lymphoma leads to earlier detection of breast cancer and presumably saves lives [360–362]. Zeng et al. [363] studied the impact of prior chest radiation therapy on breast tissue background parenchymal enhancement on screening MRI and found that enhancement was significantly higher in irradiated patients compared with age matched unirradiated controls. Breast density was not different between the 2 groups, however. The clinical significance of these findings, if any, is not clear at present. Specifically, it is not known whether women with enhancement of background parenchyma on MRI are more likely to develop secondary breast cancer.

Sarcomas, breast and thyroid carcinomas occurred with similar frequency and latency in pediatric Hodgkin lymphoma patients treated with high-dose or reduced-dose radiation therapy [355]. Melanoma and non-melanoma skin cancers have also been reported in patients treated for Hodgkin lymphoma, especially as children.

Hodgkin lymphoma patients who develop a second neoplasm are at risk for additional neoplasms [364]. Armstrong et al. studied 1382 survivors of childhood Hodgkin lymphoma who developed a second neoplasm and found that 28% developed another cancer and of those, 40% developed still another cancer or cancers [365]. The incidence of multiple new cancers was 41% in patients with one new cancer who had been irradiated for Hodgkin lymphoma compared with 26% in those who were not irradiated after 15 years of observation post Hodgkin lymphoma treatment. There may be a genetic basis for the development of a second malignancy after treatment for Hodgkin lymphoma. Best et al. [366] found two variants at chromosome 6q21 associated with second malignancies in patients who received radiation therapy as children for Hodgkin lymphoma. This finding needs further exploration especially in children who ultimately develop multiple posttreatment malignancies. Second malignancies are a significant cause of morbidity and mortality of patients treated for Hodgkin lymphoma as teenagers or young adults [367] and adults [368].

Pregnancy and Infertility

Pregnancy during remission from Hodgkin lymphoma does not increase the risk of relapse [369]. As many as 3% of all patients with Hodgkin lymphoma are pregnant at the time of diagnosis [370]. During the first trimester it is best to defer lymphoma treatment if at all possible. If not, consider single agent vinblastine therapy until the second or third trimester [370]. Combination chemotherapy is safe during the latter two trimesters. Radiation therapy should be avoided during the entire pregnancy unless absolutely necessary [370, 371].

Cancer testis antigen CT45 is expressed by Reed–Sternberg cells in 50–60% of Hodgkin lymphoma patients, which is the highest rate of all malignancies [372]. It is not known whether or not this finding has anything to do with the oligospermia found in 10–25% of patients before treatment [373, 374]. Cryopreservation of semen is routinely performed by many centers before treatment of young male patients. In one study it doubled the likelihood of posttreatment fatherhood [375]. However, this is not always feasible because many males with Hodgkin lymphoma have oligospermia or other sperm abnormalities such as DNA damage [376] at the time of diagnosis, and their sperm samples often do not meet the criteria of most reputable sperm banks [377]. Oligospermia or azoospermia prior to treatment is more common in patients with B symptoms [378].

In general, infertility is more common in male patients owing to the higher sensitivity of their reproductive organs to chemotherapy-related damage, especially that induced by alkylating agents and procarbazine. The MOPP and BEACOPP regimens [379] have induced infertility in up to 70% of male patients, whereas ABVD and other nonalkylating agent-based regimens less frequently induce infertility, which is usually irreversible for years [380]. Furthermore, gonadal damage is related to patient age at the time of treatment. In a large recent study 45% of females treated with ABVD, 35% of females treated with BEACOPP, 53% of males treated with ABVD and 33% of males treated with BEACOPP attempting conception after lymphoma treatment had children [381]. BEACOPP is significantly more toxic to ovarian function than ABVD, but in women treated with GnRH analogs during combination chemotherapy, 91% had normal ovarian function 2 years post chemotherapy [382], and women treated with BEAM and stem cell transplantation before age 40 years are significantly more likely to undergo early menopause compared with those treated with ABVD [383, 384]. Prepubertal patients carry a lower risk of infertility after treatment for Hodgkin's lymphoma [385, 386].

Sterility problems appear to be less common in adult women than in men, probably because the germ cells of the ovary with their low proliferative rate are less sensitive to drugs than are those of the testicle. Of women treated with regimens containing procarbazine or alkylating agents, most become amenorrheic [150], for years, especially those with advanced-stage disease or age over 30 years [387]. Those who use oral contraceptives may have a return of menses sooner than those who do not [387]. Infertility decreases in frequency from 1 to 10 years after chemotherapy. Reestablishment of menses and the possibility of

pregnancy in a treated woman are also correlated with age and the cumulative dose of drug exposure. However, amenorrhea is no guarantee of infertility, and patients should be made aware of that fact. Most data indicate that the risk of spontaneous abortion or fetal abnormalities in patients previously treated with combination chemotherapy is not increased compared with the risk to the general population, but there are presently no data on the risk to subsequent generations, if any. Despite the potential infertility associated with Hodgkin's disease and its treatment, it may be relatively short term in many patients. Pregnancy occurs in patients treated for Hodgkin's disease and spouses of treated male patients with surprising frequency [388]. Gonadotropin-releasing factor (triptorelin) administered monthly during chemotherapy may prevent or reduce the gonadotoxicity of chemotherapy in women receiving initial but not salvage therapy [388]. Ovarian cryopreservation may be a useful method of preserving fertility in young women prior to therapy. In a study of 24 patients, all ovarian specimens obtained were free of evidence of Hodgkin's lymphoma, even in patients with subdiaphragmatic disease [389].

Finally, some women treated with chest irradiation may not be successful at breastfeeding [390].

Thyroid Complications

Thyroid dysfunction is related to neck irradiation, and chemotherapy seems to play no role in its incidence or severity [391]. Of 1787 patients treated at Stanford, 50% exhibited thyroid damage within 20 years [392].

The median latency period from therapy for thyroid dysfunction was 4.6 years, and the most frequent dysfunction was hypothyroidism (47%), usually subclinical, followed by Graves disease (3.1%), benign alterations (3.3%), and autoimmune thyroiditis (1.3%). Others have reported similar data [393]. Furthermore, an increased incidence of thyroid cancer is reported, especially in pediatric patients [355, 394]. The risk of developing thyroid dysfunction persists for up to 25 years after treatment. For unknown reasons, white pediatric patients are 2.5 times more likely to develop hypothyroidism after irradiation for Hodgkin lymphoma than are black children [395]. Clinical examination and laboratory tests are recommended at 6 month intervals during the first 5 years and annually thereafter. Patients found to have an elevated level of thyroid-stimulating hormone following neck irradiation should be treated with thyroid hormone for the rest of their lives [396].

Renal Disease

Idiopathic minimal change nephrotic syndrome (MCNS) is an uncommon immune-mediated glomerular entity occasionally occurring in patients with classical Hodgkin lymphoma. Audard et al. [397] found that c-mip was selectively expressed in Reed–Sternberg cells of patients with Hodgkin lymphoma and MCNS but not in Hodgkin lymphoma patients without MCNS. Upregulation of c-mip may be a useful marker for MCNS.

Familial Hodgkin Lymphoma

In our databank of over 750 pedigrees of families with multiple hematologic malignancies there are 61 families with multiple cases of Hodgkin lymphoma, 69 families with both Hodgkin lymphoma and non-Hodgkin's lymphoma, 19 families with Hodgkin lymphoma and myeloma and/or Waldenström's macroglobulinemia, and 35 families with Hodgkin lymphoma and various leukemias. We recently reported on 26 families with both myeloma and Hodgkin lymphoma in their pedigrees [117] and there are several patients reported in the literature that have both B-cell neoplasms. In 90% of these pedigrees anticipation is present, which suggests a genetic rather than an environmental basis for the observation [22]. The data suggest that familial Hodgkin lymphoma may be more common than previously recognized, and that a basic commonality among most B-cell hematologic malignancies exits that is not generally appreciated. The fact that Hodgkin lymphoma in sibs more often than not occurs in sibs of the same sex led us to postulate the existence of a Hodgkin lymphoma gene at the short arm telomeres of the sex chromosomes near the SHOX locus [398]. Familial Hodgkin lymphoma is more fully discussed in Chap. 41.

Other Complications and Associations

De Bruin et al. [399] studied 2201 5-year survivors of Hodgkin lymphoma treated before the age of 51 years to determine if they had an increased risk for stroke and transient ischemic attacks. After a median follow-up of 17.5 years, 55 patients had a stroke, 31 had transient ischemic attacks and ten had both at a median age of 52 years. The standardized incidence ratio for stroke and transient ischemic attack was 2.2 and 3.1, respectively. Mediastinal and neck irradiation were independent risk factors for both and chemotherapy was not associated with an increased risk for either. Most ischemic events were caused by large artery atherosclerosis or cardioembolism. These heretofore unrecognized complications of radiation therapy need to be taken into account when deciding on therapy.

Hydrocele may develop in patients who have undergone staging laparotomy (now obsolete) or para-aortic nodal irradiation presumably due to disruption of para-aortic and/or pelvic lymphatics [400]. Small bowel obstruction may also uncommonly occur after laparotomy or abdominal irradiation [401].

Radiation to the para-aortic lymph nodes, especially if the tail of the pancreas is included in the field, increases the risk for diabetes mellitus in survivors of Hodgkin lymphoma. The incidence of diabetes is directly related to the radiation dose [402].

Chronic fatigue among survivors of Hodgkin lymphoma is significantly more common than in the general population [403, 404]. Clinical depression is strongly associated with high levels of fatigue [403], which may lead to work loss [404, 405]. Patients treated for advanced stages of Hodgkin lymphoma have poorer sexual quality of life long-term than others [406].

Adolescent and young adult Hodgkin lymphoma survivors have fewer infections during childhood than do controls. They also appear to have a deficit of rare gut microbes It is not known whether this decreased microbial diversity is due to the lymphoma, its treatment or other factors [407].

Future Considerations

New risk scores based on molecular characteristics of Hodgkin's lymphoma as well as histologic and immunologic characteristics [408] may ultimately replace those currently in use, which are based primarily on clinical characteristics. Such new methods of predicting risk may lead to more precise individualized therapy.

New data suggest there may be a role for rituximab in classical Hodgkin's lymphoma [409, 410]. Rituximab plus ABVD improved event-free survival by an average of 17% compared with ABVD alone across all prognostic groups [409]. New trials studying the efficacy of rituximab in classical Hodgkin's lymphoma are ongoing and may yield important results.

A variety of new agents not discussed above, such as lenalidomide [411], everolimus [412], and panobinostat [413] have shown clinical activity in this lymphoma that deserves further study. ABT-737, a new small molecule that inhibits the Bcl-2 family of apoptosis regulators, is active against cell lines derived from patients with Hodgkin's lymphoma [414] and will be evaluated in clinical studies. Brentuximab vedotin, as discussed above, will play a major role in the future treatment of Hodgkin lymphoma.

Finally, Blacks and Hispanics have an increased risk of death from Hodgkin's lymphoma compared with whites, at least in California. The cause is most likely poorer access to health care and/or presentation at advanced stages compared with Whites [415]. That is unacceptable in modern times.

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44 Radiotherapeutic Management of Lymphomas

John P. Plastaras and Eli Glatstein

Staging

Effective management of lymphomas first requires an understanding of the extent of the disease. This guides not only choice of therapies, but also helps define the goals of therapy. Cure of lymphoma requires elimination of all clinically evident disease, as well as regional and systemic subclinical disease. Ideally, we stage patients with cancer in general to allow for meaningful comparisons of results among countries, institutions, eras, and treatments. The staging system for lymphomas is unusual in that it favors the techniques of the radiation oncologist, rather than the surgeon, which is true for most neoplasms. The difference between Stage II and III is often a difference in what can be easily encompassed within radiation fields. Peters noted improved survival in patients treated with radiation to both involved and non-clinically involved sites [1]. Today radiotherapy can be delivered to a wide range of volumes with a range of doses, allowing for tremendous flexibility. The choices made by the radiation oncologist depend on both the probability of subclinical involvement and probability of disease control. This requires careful assessment of clinical factors, disease extent, and knowledge of the patterns of relapse.

The staging of lymphomas has evolved over time, most notably by advances in imaging. At the time of the development of curative regimens for lymphomas, lymphangiography and plain radiography had been an important complement to the physical exam. The advent of staging laparotomy with splenectomy for HL was for a time an important method to detect subdiaphragmatic disease [2]. Body computed tomography (CT) scanning had been a standard for some time for staging [3]. Although Gallium-67 scanning enjoyed some popularity for a time, especially as a predictor of response, it has been replaced by ^{18}F-fluorodeoxyglucose (FDG) positron emission tomography (PET). FDG-PET, first used for lymphoma in 1987, is not only sensitive in lymphoma, but also is prognostic [4–6]. In lymphoma, FDG-PET upstaging and downstaging changes management (18% in early-stage HL), and has an estimated sensitivity of 90% and specificity of 91% [7, 8]. It has become a standard staging study for HL and aggressive NHL, and in fact, the standard definitions of response, including complete response (CR) is now described in terms of PET uptake [9]. The five-point Deauville scale is now being used for both interim and final treatment response assessment [10]. Due to the evolving practices of staging, historical eras need to be considered.

Dose–Response

Increasing doses of either radiation or chemotherapy result in increasing tumor cell kill and normal tissue toxicity. Kaplan demonstrated that for HL, there is a clear dose response with radiation alone. Doses as low as 10–20 Gy can control about 50% of involved sites, but to achieve >98% local control, 44 Gy was needed regardless of size [11]. The Kaplan data included all sizes and all patient as there was no curative chemotherapy at the time (Fig. 44.1) More modern series with modern radiotherapy techniques suggests that somewhat lower doses can achieve similar results, but larger masses require higher doses [12, 13]. The newer data which support lower doses include many patient who received multiagent chemotherapy as well. NHL, which generally have more malignant cells per volume of tumor than HL, generally require higher doses [14]. It is intuitive that a greater number of tumor cells requires higher dose, therefore it is important to think logarithmically if the goal is to reduce the number of malignant cells to statistically less than 1.

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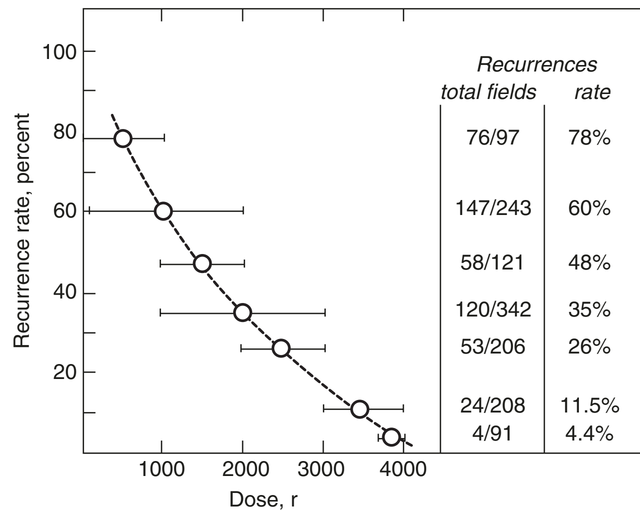

Fig. 44.1 Composite data on recurrence rate as a function of dose, with recurrence rates plotted to the midpoint of dose ranges indicated by the horizontal bars and brackets (reproduced with permission from the American Association for Cancer Research. From Kaplan HS. Evidence for a tumoricidal dose level in the radiotherapy of Hodgkin's disease. Cancer Res 1966;26(6):1221–4)

Unfortunately, normal tissues are also sensitive to dose, which establishes the principle of establishing a therapeutic index. Both acute and especially late effects need to be considered. Hematologic toxicity is the predominant factor that limits not only the maximum tolerated single chemotherapy cycle dose, but also the number of cycles that can be given. Cumulative chemotherapy doses can increase the risk of late side effects, but it is more challenging to adjust the doses during treatment. For example, increasing doses of doxorubicin correlate with depressed left ventricular function, even when the cumulative doses were below the accepted "threshold" level [15, 16]. There is an association between the risk of leukemia and cumulative chemotherapy dose following treatment for HL, especially for alkylating agents [17]. With radiation, the dose–response for late toxicity is complicated by the fact that dose per fraction increases late complications in addition to the total dose. Furthermore, because radiation effects on normal tissues only occur within the treated fields, knowledge of what organs were treated is also important. Dose-related toxicities with radiation include thyroid dysfunction, pneumonitis, pulmonary fibrosis, xerostomia, soft tissue hypoplasia in children, and valvular and coronary artery disease [18–25]. The dose that is ultimately given is defined by the minimum dose required to control all tumor sites with the fewest and least severe side effects. The doses required will vary with the age of the patient, the tumor volume, the amount of chemotherapy in the regimen, and comorbid conditions.

Combined-Modality Therapy

Tailoring of Local–Regional Therapy

A major premise in lymphoma management is that the amount of treatment should be adjusted to the risk of treatment failure because of concern about acute and late toxicity. Chemotherapy may be tailored by adjusting the number of agents, duration or number of cycles of chemotherapy, total dose of individual drugs, and the frequency and time interval over which agents are given. What cannot be accomplished with chemotherapy is anatomically distinct dose variation according to tumor burden. With radiation therapy, fields can be selected that treat only volumes that are at risk to be involved by lymphoma. Within a treatment field, critical normal tissues can be partially or totally excluded from radiation exposure. A modest dose reduction of only 10% has major implications in terms of minimizing morbidities. Differential doses can be delivered to differently targeted tumor volumes through customized blocking, selection of radiation energy, manipulation of the direction of entry of the radiation beam, and field size reductions during the course of therapy. Advances in imaging have allowed improved radiation treatment planning. CT-based radiation treatment planning allows for cross-sectional confirmation that target nodal sites are adequately covered by radiation fields [26–28]. CT planning decreases the risk of inadvertently shielding target volumes compared to plain film planning, which may have resulted in "marginal misses" in the past [29]. Advances in the physics of radiotherapy have allowed the treatment of irregular target volumes to high doses while sparing nearby critical structures. Intensity modulated radiotherapy (IMRT) uses computer-based algorithms and automated control of adjustable "multileaf collimator" blocking to sculpt radiation doses around structures like the parotid gland, heart, or lungs. IMRT may be appropriate for certain patients with mediastinal malignancies, but the concern of spreading low dose to large volumes may be of concern when considering the possibility of second malignancies [30, 31]. Proton radiotherapy, which has no exit dose, may also be a useful technique for minimizing late toxicities in lymphoma patients, especially young women with mediastinal involvement [32].

Operator Dependence

It is not disputed that the skill and experience of a surgeon and their team can dramatically impact outcomes, especially if the procedure is complicated. A similar phenomenon is observed with radiotherapy in lymphoma where outcomes vary with experience, skill, era, and equipment [33, 34]. The

prediction of the extent and location of volumes at risk, design of radiation treatment plans, and the actual delivery of the intended dose varies with the subspecialty expertise of the physicians, medical physicists, dosimetrists, and radiotherapy technicians. Quality assurance in radiotherapy has received considerable attention, although many of the same issues are relevant in the optimal delivery of chemotherapy.

Patterns of Failure

After chemotherapy alone, the majority of treatment failures occur in sites of initial involvement, especially large volume disease [35–37]. After radiotherapy alone, the great majority of treatment failures occur outside of the radiation fields [38]. Complementary patterns of failure establish the rationale of improved efficacy with CMT, as well as the decrease in morbidity resulting from truncating both chemotherapy and radiation therapy.

Potential Advantages of Combined-Modality Therapy

If the only goal of CMT were improved efficacy, one would simply add radiotherapy on top of a full course of chemotherapy. This would result in all of the side effects of both treatments, with the added possibility of supra-additive toxicity with a marginal additional benefit. A basic principle of cancer treatment is to use the least amount of therapy to achieve efficacy. With the complementary efficacy based on patterns of failure and complementary non-overlapping toxicities, less intense chemotherapy and less intense radiotherapy may be combined to maximize efficacy while minimizing toxicity. The challenge has been to determine how much radiation can substitute for how much chemotherapy and in what settings. The optimal combination of the elements in CMT will likely vary depending on the stage, likely extent of subclinical disease, bulk, patient-related factors, and response assessment.

Risk- and Response-Adapted Therapy

Fortunately, because our treatments are effective for many NHL and the majority of HL patients, late treatment effects must be considered while maintaining high cure rates [39]. There are many permutations of how chemotherapy and radiation are blended, including the number of drugs, number of cycles, radiation volumes, and doses. We know that we overtreat many patients, but in general, we don't really know which ones. Risk-adapted therapy attempts to predict how much and what kind of therapy each patient needs at the outset. Response-adapted therapy uses information obtained after some therapy has been given to tailor therapy. Historically, risk-adapted therapy has relied on basic predictive clinical and laboratory factors. For example, in most cooperative group trials, patients with HL are grouped into at least three, sometimes four, risk groups based on stage, number of involved sites, bulky disease, age, B symptoms, and/or ESR: unfavorable, intermediate, favorable, and, sometimes, very favorable [40–42]. There is less clarity about the groupings in NHL, but these have focused on stage, bulk, and the varying forms of the International Prognostic Index (IPI). Within these risk categories, decisions about radiation take into account the volume of clinically evident disease and the probability of subclinical disease. Historically, the types of radiation fields varied from total nodal (mantle and "inverted Y" fields), subtotal nodal, extended field, and involved-field radiation. Today "involved node" and "involved site" radiation therapy are currently used, which incorporate pre-chemotherapy imaging, response assessment, and 3D volume-based radiation planning (see below). The use of radiation may be used only in certain scenarios, such as with bulky disease or a limited number of involved nodal sites. For HL, the results of clinical studies support an algorithm to tailor the number of cycles of chemotherapy and the volume of radiation based on risk grouping and site/size of involvement [38]. Response-adapted therapy has gained increasing acceptance, especially in HL. Conceptually, an early test of responsiveness to treatments is likely to be a strong predictor of long-term outcome that will trump other prognostic risk factors. The challenge has been to identify the appropriate early test. Shrinkage of masses on CT scanning has been unsatisfactory, resulting in terms like "Complete Response, Unconfirmed." This is due to the fact that a lymphoma mass is not entirely composed of malignant cells, and even if the mass is sterilized, some enlargement may remain on cross-sectional imaging. Functional imaging with nuclear isotopes may overcome this issue. Although early work with gallium scanning was used to predict results after chemotherapy, FDG-PET is now the standard for response-adapted therapy. The definition of a complete response by the Lymphoma Working Group now includes complete resolution of metabolic uptake by FDG-PET. This definition is justified by convincing data that disappearance of FDG uptake after chemotherapy predicts long-term outcomes [43–45]. In HL, PET response after 2 cycles of ABVD overshadows other prognostic factors [46]. Although FDG-PET is a powerful imaging biomarker in HL, omission of RT in early responders has been shown to be nonequivalent to CMT in two separate large trials. The UK RAPID trial randomized non-bulky HL patients after ABVD × 3 with Deauville 1 and 2 (less than mediastinal blood pool) responses to no further

treatment or IFRT [6]. Designed as a non-inferiority study, omission of RT was "not non-inferior" by both intention to treat and per protocol analyses. Similarly, the EORTC/LYSA H10 trial had to close the non-radiation containing arms in both favorable and unfavorable arms that used a response-adapted design after ABVD × 2 [47]. Ongoing trials are still exploring how best to use this powerful imaging biomarker, despite its inability to predict which patients can avoid consolidative RT in limited-stage HL.

Hodgkin Lymphoma

Today, much is understood about the role of radiotherapy as monotherapy for HL. With the success of treatment of HL in the 1960s and 1970s with radiotherapy alone, long-term follow-up has taught us many lessons about late radiation effects. Over time, multi-agent chemotherapy has become the backbone to cure classic HL. Today, only limited-stage nodular lymphocyte-predominant (non-classic) HL is commonly treated with radiotherapy alone. For classic HL, current research attempts to define the optimal use of radiotherapy in the context of CMT.

Evolution of Field Design and Involved Site Radiotherapy

The International Lymphoma Radiation Oncology Group (ILROG) has recommended that "modern" radiotherapy for lymphoma, termed "involved site radiotherapy" (ISRT), use small volumes without elective nodal radiation with image-based treatment planning customized to each patient's unique anatomy [26–28]. The EORTC/GELA have adapted a stricter protocol-based version of limited volume radiation termed "involved node radiotherapy" (INRT) [48]. This requires that patients have their initial PET/CT scans in the radiotherapy treatment position and that intravenous contrast be used in radiation treatment planning CT scans [49]. The adoption of ISRT and INRT into routine practice did not happen until around 2013 when it was recommended by the NCCN and the ILROG's first guideline to ISRT was available. However, to understand the history of lymphoma treatment and the majority of the published literature, an appreciation for historically used radiation fields is critical. Previously, standardized fields incorporated groups of nodal and extranodal lymphoid areas in modular units largely based on bony landmarks. These standard modules included the Waldeyer ring, neck, axillary, mediastinal, spleen, para-aortic, inguinal-femoral, and pelvic areas. Involved-field radiotherapy (IFRT) used standard field borders to electively treat limited nodal groups surrounding regions of disease [50]. For example, a standard neck field would have been used for a single midjugular node, a single supraclavicular node, or a chain of nodes extending from the base of the skull to the clavicle. Combinations of involvement were covered in specialized fields such as the mantle (neck, axillae, hilae, and mediastinum) or the inverted Y (femoral, inguinal, pelvic, and para-aortic areas). Treatment of additional uninvolved nodal groups contiguous to IFRT borders was called extended-field radiotherapy (EFRT). Subtotal nodal irradiation (STNI) included the mantle, para-aortic, and spleen fields and had been considered standard extended-field therapy for stages I and II supradiaphragmatic HL. Total nodal irradiation also included pelvic irradiation. The majority of these fields were treated with simple field arrangements, namely anterior-posterior/posterior-anterior fields.

With the advent of CT imaging and three-dimensional treatment planning systems, at risk volumes could be better defined and targeted with more complex beam arrangements. It is clear that many treatment failures in the past were related to inadvertent shielding of gross tumor [29, 52]. In radiotherapy for other malignancies, image-based treatment planning customized to each patient's unique anatomy have replaced standardized fields. In HL however, recently published cooperative groups studies still employed standardized IFRT, including the German Hodgkin Study Group (GHSG) HD10, HD11, and HD13 studies. Although the GHSG is testing involved-field (which includes elective nodal RT) versus involved nodal radiation in the HD17 trial, volume-based planning has replaced the use of bony landmarks alone for radiotherapy planning [53]. As mentioned above, the ILROG has already endorsed omission of elective nodal irradiation in the case of chemosensitive disease [26–28]. The combination of targeting much smaller target volumes with highly conformal techniques (IMRT, proton therapy) should lead to dramatically lower radiation-related toxicity in the modern era as demonstrated in Fig. 44.2.

Early-Stage Disease

Prior to the era of multiagent chemotherapy, it was discovered that radiotherapy alone with extensive fields (STNI or total nodal irradiation) could cure early-stage HL in adults 75–80% of the time. However, cure rates could be improved by adding multiagent chemotherapy, such as doxorubicin–bleomycin–vinblastine–dacarbazine (ABVD), to large-field radiation [38, 54]. The evolution of clinical trials for HL demonstrated that CMT with smaller fields was superior to large-field radiation alone, which then lead to the question of whether radiotherapy could be omitted altogether [55]. An NCI Canada/ECOG study stratified 399 patients into favorable or unfavorable groups and randomized them to either ABVD alone (4–6 cycles depending on response) or a regimen containing radiation (favorable group received STNI

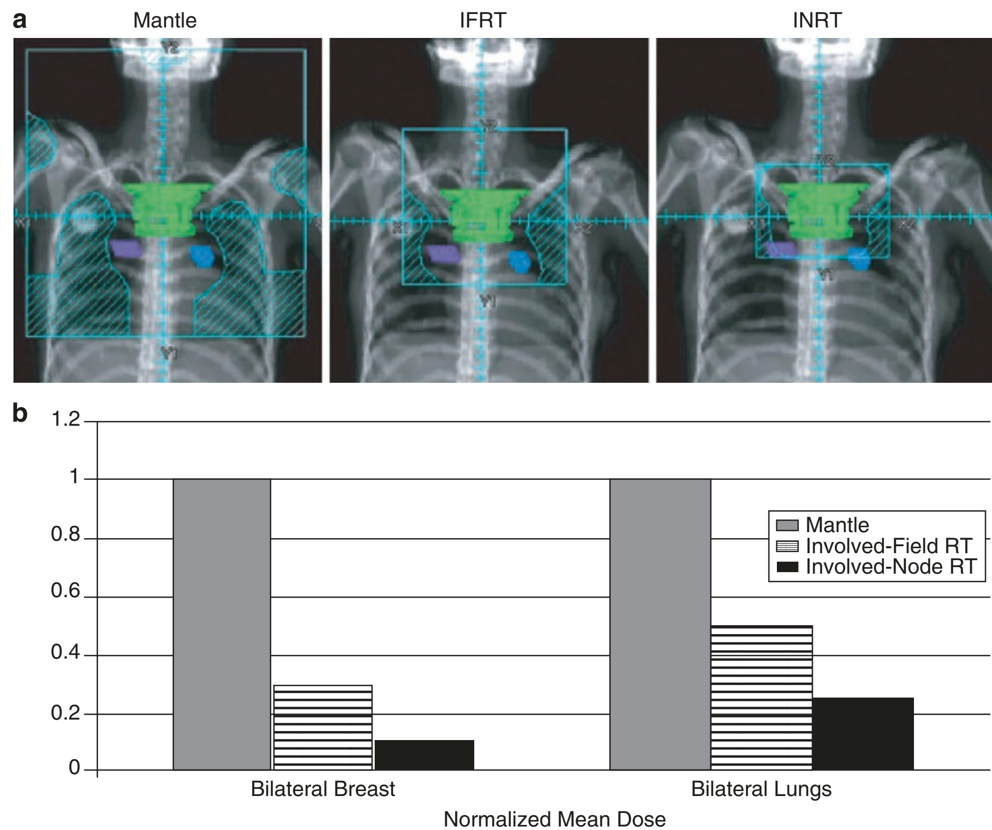

Fig. 44.2 (**a**) Representations of fields for a historic mantle field, IFRT, and INRT (similar to ISRT). Postchemotherapy target volume is shown in *green*, hila are shown in *blue* and *violet*. (**b**) Proportional reduction in breast and lung dose using the three techniques. Reproduced with permission from Hodgson et al. [51]

alone; unfavorable group received two cycles ABVD then STNI) [56]. Freedom-from-disease progression at 12 years was superior in those patients receiving radiation (92% vs. 87%, $p = 0.05$). However, overall survival was worse among those receiving STNI (87% vs. 94% $p = 0.04$), with excess deaths coming from second cancers and other causes in the STNI group. Although the radiotherapy used in this study was well outdated by the time the long-term results were published in 2012, the favorable outcomes in the ABVD only arm have been used as an argument against the need for CMT. The EORTC/GELA H9F trial used a reduced intensity chemotherapy regimen (EBVP × 6 cycles) with no radiation, 20 Gy, or 36 Gy involved-field radiation. The chemotherapy only arm had to be stopped due to excessive relapses [57].

In favorable risk HL patients, fewer cycles of chemotherapy and lower doses of radiation may still achieve disease control. The GHSG HD10 study compared 2 vs. 4 cycles of ABVD and 20 Gy vs. 30 Gy IFRT [58]. There was no significant difference between 4 × ABVD and 2 × ABVD in terms of overall survival at 5 years (97.1%; vs. 96.6%) or freedom from treatment failure (93.0% vs. 91.1%). In terms of radiation dose, there were no significant differences between 30Gy and 20Gy IFRT in terms of overall survival (97.6% vs. 97.5%) or freedom from treatment failure (93.4% vs. 92.9%), which has led to the adoption of ABVD × 2 and 20 Gy as an acceptable option for very favorable patients. Unfortunately, omission of dacarbazine or bleomycin from ABVD × 2 is not equivalent to the full four-drug regimen as shown in the HD13 [59].

In unfavorable risk early-stage patients, the GHSG HD11 study compared 4 cycles of ABVD with 4 cycles of BEACOPP (standard) as well as 20 Gy vs. 30 Gy IFRT [60]. Disease control was equivalent with ABVD with less toxicity and 30 Gy was superior to 20 Gy in the context of ABVD, so ABVD × 4 with 30 Gy became the standard for unfavorable risk early-stage patients.

The Stanford V chemotherapy regimen uses seven drugs (doxorubicin, bleomycin, nitrogen mustard, vinblastine, vincristine, etoposide, and prednisone) given in a dose-dense and intense fashion over a short period of time, supplemented with involved-field radiation therapy to all sites with bulky involvement (tumors at least 5 cm in transverse dimension, macroscopic lesions in the spleen, or massive mediastinal disease) [61]. This regimen uses lower doses of each agent to maximize efficacy with less toxicity. Long-term results in early-stage patients with bulky disease as well as advanced disease are excellent, adding this regimen as alternative to ABVD [62].

In growing children, the doses of radiation traditionally used in HL (30–40 Gy) may result in significant musculoskeletal hypoplasia, therefore, efforts to limit radiotherapy have been intense among the pediatric group. The Pediatric

Oncology Group (POG) compared 6 months of MOPP–ABVD chemotherapy with 4 months of the same chemotherapy and low-dose (25.5 Gy) IFRT therapy in children with stage I-IIIA HL, showing that radiotherapy could substitute for 2 months of chemotherapy [63]. The AHOD0031 study assigned treatment groups based on early response assessment following two cycles of doxorubicin, bleomycin, vincristine, etoposide, cyclophosphamide, and prednisone (ABVE-PC) [64]. "Rapid early responders" who achieved complete response were randomized to 21 Gy of IFRT. Event-free survival at 4 years was not significantly better with IFRT compared to no RT (87.9% vs. 84.3%, $p = 0.11$). In that trial, all "slow early responders" were given 21 Gy IFRT, but they found that those who were PET-positive at response assessment benefited from intensified chemotherapy.

Although rare, nodular lymphocyte-predominant HL should be considered differently from classic HL. It usually presents as limited stage, and in these very favorable patients the risk of extensive subclinical disease may be judged so minimal that treatment aimed at only the clinically evident disease may be warranted [39, 65]. Pediatric patients with IA lymphocyte-predominant HL located in a peripheral site such as the upper neck, axilla, or femoral nodes have frequently been treated with only involved-field radiotherapy [66]. Even very low dose radiation (4 Gy) can even result in durable responses in some patients with nodular lymphocyte-predominant HL, although higher doses (30–36 Gy) are more standard [67]. When using radiation alone, "involved site" radiotherapy for nodular lymphocyte-predominant HL does involve electively treating adjacent lymph node groups (ILROG HL ref).

Stage IIB Bulky and Advanced-Stage Disease

Patients with stage IIB bulky disease have been included with other Stage III/IV patient in clinical trials; however, CMT in IIB bulky HL results in very good outcomes in the modern era [68]. In both Stage IIB bulky and III/IV HL, chemotherapy is the primary effective treatment, but radiotherapy can be more easily incorporated in Stage IIB bulky patient. The role of radiation therapy in patients with Stage III/IV disease is less clear, but may be of use in select patients, namely those with a partial response (PR) after chemotherapy.

The EORTC employed response-adapted scheme patients where stage III/IV patients were treated with MOPP-ABV for 6–8 cycles depending on the response after 4 cycles. If patients had a CR after 4 or 6 cycles, they were randomized to either IFRT to 24 Gy or observation. If they only had a PR after 6 cycles, no further chemotherapy was given and instead IFRT was given in a non-randomized fashion [69]. For the randomized patients with CRs, there was no difference in 5-year event-free or overall survival rates. The non-randomized PR patients who all received IFRT had event-free and overall survival rates similar to the CR randomized arms [70]. Thus, the role of radiation in advanced-stage patients has been primarily limited to those who do not achieve a CR.

Alternate chemotherapy regimens for advanced-stage disease have been studied. For high risk patients with advanced disease, dose escalated BEACOPP appears to have better results as reported in the GHSG HD9 trial, which has led to this being the reference therapy on the subsequent GHSG trials [71]. The HD15 study only used radiation in advanced-stage patients with FDG-avid residual masses >2.5 following BEACOPP [72]. The use of the Stanford V regimen in advanced-stage disease employs radiation to sites of initially bulky disease to 36 Gy [62]. Omission of radiation when using Stanford V results in poorer outcomes, emphasizing the important role of radiation in this abbreviated chemotherapy regimen [73]. In our opinion if the chemotherapy regimen has be altered, resulting in deviation from recommended agents, doses, as schedule, radiation therapy should be considered as additional adjuvant.

Surveillance for Late Effects

It is clear that survivors of HL are at increased risk for cardiovascular disease and second malignancy. The risk of cardiovascular disease appears to be at least partially related to radiation dose, specifically related to the mean heart dose [19, 20, 22, 74]. The rate of second solid malignancies in the HL survivors increases with the interval after irradiation, with the highest risk when treatment occurs at an early age [75]. Breast cancer seems to be most significantly increased in women who received radiation during their early teenage years to higher doses [76]. The use of smaller radiation volumes has resulted in lower risk of second malignancies, however it appears that the increased risk of second cancers is intrinsic to the diagnosis of HL, regardless of the era of diagnosis [77, 78]. More conformal techniques, such as proton therapy for female patients with mediastinal disease, may lower the risk of second cancers [79]. Breast cancer screening with mammography alone may not be sufficient to detect breast cancer in female HL survivors, and breast MRI will likely play an important role in close surveillance [80]. Lung cancer is also a common problem in HL survivors who are smokers; it is rare in nonsmokers, therefore smoking cessation counseling is crucial [81]. The coordination of surveillance for cardiac disease, second malignancies, and endocrine abnormalities requires ongoing follow-up in HL survivors who may benefit from comprehensive survivorship care plans [82].

Non-Hodgkin's Lymphoma

The natural history of NHL has been more difficult to understand than that of HL, making various therapeutic approaches hard to evaluate. Part of the difficulty has been to categorize the spectrum of diseases falling under the rubric of NHL. It is useful for clinical purposes to consider the management of the NHLs in three categories: the low-grade, the intermediate-grade, and the high-grade lymphomas. Because the large majority of NHLs are B-cell lymphomas carrying the CD20 marker, the advent of rituximab has probably been the single most important advance in treating all stages of disease [83]. Radioactive isotopes bound to anti-CD20 monoclonal antibodies have produced excellent tumor responses [84].

Low-Grade Lymphomas

Stages I and II

In the advanced-stage low-grade lymphomas, there has been no convincing evidence of cure with multiagent chemotherapy [85, 86]. Radiation therapy, however, in early-stage low-grade lymphomas has resulted in approximately 50% freedom from relapse and survival rates at 10 years in clinically staged patients [87]. Although this cure rate is probably even better with modern staging, only a minority of patients stage I, low-grade lymphoma are initially treated with radiotherapy [88, 89]. Contrary to National Comprehensive Cancer Network treatment guidelines, patients are often only observed or given rituximab [88]. Relatively modest doses (24 Gy) are considered optimal for limited-stage low-grade lymphomas based on randomized data, so toxicity is low, even when sensitive regions need treatment, like the abdomen/pelvis [90]. The majority of relapses after regional radiation are out of the radiation field, usually in other nodal or lymphoid sites, depending on histologic subtype [91]. The addition of chemotherapy (and in particular immunochemotherapy) to radiotherapy has a DFS benefit but no overall survival benefit [92].

Advanced Stage

In stage III low-grade lymphomas with limited involvement, 40–50% of patients appear to be free from disease at 10 years after comprehensive lymphatic irradiation [93–95]. Concerns for long-term toxicity, however, has resulted in the use of systemic chemotherapy in this group of patients instead, in particular single agent rituximab as first line therapy. A promising but rarely used initial treatment of advanced stage of follicular lymphoma is radioimmunotherapy [96]. Unlike other treatments for advanced-stage low-grade lymphoma, radioimmunotherapy may have a tail on the survival curve [97]. In addition to producing long-term disease-free intervals in patients with stage I, II, or "early" stage III disease, radiation therapy also has value as a palliative agent. Very low dose radiation (4 Gy) provides frequent and sometimes durable palliation with minimal toxicity [98, 99].

Intermediate-Grade Lymphoma

The role of radiation in intermediate-grade NHL in adults has been studied by several prospective randomized trials. The Eastern Cooperative Oncology Group (ECOG) randomized patients who had a complete response after eight cycles of cyclophosphamide, doxorubicin, vincristine, and prednisone (CHOP) chemotherapy to no further therapy or involved-field radiation to 30 Gy [100]. Patients with a PR received involved-field radiotherapy to 40 Gy. There was a significant improvement in rates of both disease-free survival and local control with the addition of radiation therapy in the complete responders, but no survival difference. Patients who received irradiation after PRs had disease-free and overall survival rates similar to those of the complete responders who did not receive irradiation. During the same time interval SWOG compared three cycles of CHOP chemotherapy and involved-field radiotherapy with eight cycles of CHOP chemotherapy [16]. At the initial report, progression-free survival rates were similar for the two treatment arms, but the survival rate was better in the radiation therapy arm because of excess cardiac mortality attributed to the additional cycles of chemotherapy [101]. When updated, the 7-year failure-free survival curves and the 9-year overall survival curves overlapped. Excess late relapses and deaths in CHOP × 3-RT arm appeared to have negated the initial benefit, but this occurred more frequently in patients with adverse risk factors. In favorable risk patients, CHOP × 3 + RT remains a standard treatment for Stage I and non-bulky Stage II. In the GELA LNH93–1 trial, patients <61 years with localized Stage I or II aggressive lymphoma with no age-adjusted IPI adverse features were randomized to: (1) CHOP × 3 + involved-field RT to 39.6 Gy or (2) chemotherapy alone with dose-intensified doxorubicin, cyclophosphamide, vindesine, bleomycin, and prednisone (ACVBP) plus sequential chemotherapy consolidation (MTX, leucovorin, etoposide, ifosfamide, Ara-C) [102]. At 5-years the ACVBP arm had improved event-free survival (82% vs. 74%) and overall survival (90% vs. 81%). This study demonstrated that very intensive chemotherapy may improve outcomes in younger patients, although that particular regimen is rarely used. The RICOVER-60 trial has shown in a non-randomized but prospective trial that the use of consolidative radiotherapy in

patients with initially bulky disease (≥7.5 cm) is associated with improved survival [103]. The currently unpublished UNFOLDER trial enrolled bulky patients with the same size criteria, but had to prematurely halt non-radiation containing arms due to excessive events, hinting at the importance of radiotherapy in DLBCL patients with bulky disease. Other indications for consolidative radiation after full-course chemotherapy include partial responders and those with skeletal involvement [104].

Amplification of c-MYC along with either BCL-2 or BCL-6, so-called double hit DLBCL have a much worse prognosis, but it is unclear if the resistance to R-CHOP chemotherapy also extends to radio-resistance. This is an emerging area of interest, but we have leaned towards using higher radiotherapy doses in these patients when safe.

High-Grade Lymphoma

Presently, there are no convincing survival data that CMT is beneficial in Burkitt or lymphoblastic NHL, despite frequently (but often transient) responses. If radiotherapy is to be used for these uncommon lymphomas, it is probably best to use twice daily radiation since the cells have an especially high growth fraction and a very short cell cycle. This will allow exploitation between fractions notably of repopulation but also cell redistribution with the cell cycle.

Summary

Radiation is an effective tool in both HL and NHL. In HL, its optimal role may be in CMT regimens except at the most favorable end of the prognostic spectrum, where it may be used as monotherapy. Problems with quality assurance in the past have confounded our understanding of optimal radiation doses and treatment volumes as well as the potential value of radiation either as monotherapy or as an integral part of CMT regimens. Developments in imaging and treatment planning with the advent of involved node and involved site radiotherapy should decrease the toxicity of radiation therapy in the future. The role of radiation therapy in CMT regimens for early-stage intermediate-grade lymphomas is well established, but questions remain regarding optimal dose and treatment volume and the potential value of radiation therapy in more advanced disease. In low-grade lymphoma, radiation therapy currently appears to hold the greatest promise for durable, perhaps permanent, disease control, with appropriate radiation treatment volumes. Imaging biomarkers, namely FDG-PET/CT has dramatically impacted the use of radiotherapy for lymphomas, allowing for smaller more targeted fields as well as informing who will most benefit from localized treatment.

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45 Diagnosis and Treatment of Non-Hodgkin's Lymphoma of Adults

David G. Crockett, James O. Armitage, and Julie M. Vose

Introduction

The non-Hodgkin's lymphomas (NHL) represent a diverse group of neoplasms with many different clinical presentations, associated laboratory abnormalities, responsiveness to treatment, and clinical outcome. The cell of origin and stage of maturation arrest prior to uncontrolled proliferation are important to the understanding of this diversity (Table 45.1). In the USA, the incidence of NHL steadily increases from childhood through age 80 years; unlike Hodgkin's disease, no peak incidence exists for young adults. NHL is the seventh most common malignancy in men and women, respectively, and is among the most rapidly increasing malignancies [1]. They represent the seventh most common cause of death from cancer in the USA, with the age-adjusted incidence being about three times that of Hodgkin's disease [1]. The incidence of non-Hodgkin's lymphoma is twice as frequent among whites as blacks, and there is an almost 50% increase in presentation in males compared with females. The reason for this gender and race predominance is unknown; however, it may reflect a different pattern of occupational exposure or a difference in the immune status of males compared with females. Links among agricultural work (organochlorine-based pesticides), wood products, and organic solvents have been established [2].

According to Surveillance Epidemiology and End Results (SEER) data, the incidence rose approximately 3–4% per year in the 1970s and 1980s and has stabilized at 1–2% per year growth with the greatest rise observed for diffuse large B-cell lymphoma (DLBCL) in the elderly [3]. The exact reasons for these increases are currently unclear; however, there are accepted associations. Immune suppression and stimulation are important cofactors for the development of NHL [4–6], and these may be in the form of inflammatory, infectious, or neoplastic etiologies. Immunodeficiency predisposes to the increased incidence of Epstein–Barr virus (EBV)-associated NHL. Equally important is the potential role of chronic immune stimulation in autoimmune diseases such as rheumatoid arthritis or Sjögren's syndrome [7, 8]. Secondary immune suppression associated with treatment of autoimmune diseases may also contribute to pathogenesis of NHL; however, this is difficult to separate from the immune stimulation from the underlying autoimmune disease.

Other relationships exist for *H. pylori* and mucosa-associated lymphoid tissue (MALT) lymphoma, hepatitis C and splenic lymphoma, or Borrelia infection and cutaneous MALT lymphoma [9, 10]. Viruses integrate into the genome to alter gene expression or proliferation and may function as cofactors in the development of NHL. EBV has a direct role in immunocompromised patients due to HIV, posttransplant lymphoproliferative disease, and Burkitt's lymphoma (BL) [11, 12]. Human T-cell leukemia/lymphoma virus-1 (HTLV-1) has a direct causative role in adult T-cell leukemia/lymphoma, with a 5% lifetime risk in carriers. Human herpes virus 8 (HHV-8) has been associated with primary effusion lymphoma [13].

More than three decades of discoveries in the areas of immunology, histochemistry, and molecular biology have advanced our understanding of these complex diseases. NHL is characterized by a monoclonal expansion of lymphoid cells, excluding the primitive precursors that yield acute lymphoblastic leukemia and plasma cells that yield multiple myeloma. The malignant cell for most lymphomas can be traced to a specific stage in lymphoid maturation; NHL most commonly derives from mature B cells of germinal center origin, but may also include T cells or NK cells. With the advent of modern therapy based on these new data, patients with non-Hodgkin's lymphoma can now frequently be treated successfully to alter the natural history of the disease. This chapter outlines the clinical presentations,

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Table 45.1 Non-Hodgkin lymphoma histologic types

Lymphoma type	Relative incidence	Histopathology	Cell markers
Precursor T-cell leukemia/ lymphoma	40% of lymphomas in childhood	Lymphoblasts with irregular nuclear contours, condensed chromatin, small nucleoli, and scent cytoplasm without granules	TdT, CD2, CD7
Follicular lymphoma	40% of lymphomas in adults	Small "cleaved" cells (centrocytes) mixed with large activated cells (centroblasts). Usually nodular ("follicular") growth pattern	CD10, surface Ig
Diffuse large B-cell lymphoma	40–50% of lymphomas in adults	Variable. Most resemble B cells of large germinal centers. Diffuse growth pattern	CD10 and surface Ig
Mantle cell lymphoma	3–4% of lymphomas in adults	Lymphocytes of small to intermediate size growing in diffuse pattern	CD6
B-cell chronic lymphocytic leukemia/lymphoma	3–4% of lymphomas in adults	Small resting lymphocytes mixed with variable number of large activated cells. Lymph nodes are diffusely effaced	CD6, surface immunoglobulin
MALT lymphoma	~5% of lymphomas in adults	Variable cell size and differentiation. 40% show plasma cell differentiation. Homing of B cells to epithelium creates lymphoepithelial lesions	CD5, CD10, surface Ig
Burkitt's lymphoma	<1% of lymphomas in the USA	Round lymphoid cells of intermediate size with several nucleoli. Starry-sky appearance by diffuse spread with interspersed apoptosis	CD10, surface Ig
Mycosis fungoides	Most common cutaneous lymphoid malignancy	Usually small lymphoid cells with convoluted nuclei that often infiltrate the epidermis, creating Pautrier micro-abscesses	CD4
Peripheral T-cell lymphoma not-otherwise-specified	Most common T-cell lymphoma	Variable. Usually a mix of small to large lymphoid cells with irregular nuclear contours	CD3
Nodular sclerosis form of Hodgkin's lymphoma	Most common type of Hodgkin's lymphoma	Reed–Sternberg cell variants and inflammation. Usually broad sclerotic bands that consist of collagen	CD15, CD30
Mixed-cellularity subtype of Hodgkin lymphoma	Second most common form of Hodgkin's lymphoma	Many classic Reed–Sternberg cells and inflammation	CD15, CD30

diagnostic and staging evaluations, prognostic factors, and therapeutic considerations for the various types of non-Hodgkin's lymphomas.

Clinical Presentation

The clinical presentation of NHL is tremendously variable and may masquerade as any known signs or symptoms. Most patients with NHL come to medical attention because of adenopathy or constitutional symptoms; however, other problems such as abdominal fullness caused by splenomegaly, pain in involved sites of disease, anemia, infection, skin manifestations, neurologic symptoms, or systemic symptoms such as fever, night sweats, or weight loss can occasionally be the first signs of disease. These constitutional manifestations are usually the result of cytokines and chemokines that are produced by the lymphoma cells as well as the host microenvironment. The childhood NHL often differ in presentation from their adult counterparts. Childhood lymphomas have a propensity for early widespread dissemination with more frequent central nervous system (CNS) and peripheral blood dissemination. Follicular lymphomas are very rare in children, and while most childhood lymphomas are B cell in origin, there are an increased number of T-cell cases compared with adults.

The clinical features of untreated patients are often typical for the individual histologic subtype of NHL. For example, patients with follicular lymphomas most often present with painless adenopathy in the cervical, axillary, or inguinal regions. In some patients, large retroperitoneal or mesenteric lymph nodes result in symptoms that bring the patient to a physician. Even though the patient may appreciate only one or a few enlarged lymph nodes, examination and evaluation often reveal widespread lymphadenopathy. The nodes may have been present for years with either fluctuation in size or gradual enlargement. Patients with follicular lymphomas often have splenomegaly, which is especially prevalent in the later stages of the disease. Bone marrow involvement is present more frequently in nodular lymphomas than in diffuse non-Hodgkin's lymphomas. For example, one study showed a 24% incidence of bone marrow involvement with follicular lymphomas compared with a 14% involvement with diffuse lymphomas [14]. Other studies have shown up to a 50% incidence of bone marrow involvement in follicular lymphomas [15]. Occasionally patients with bone marrow involvement will also present with peripheral blood involvement; however, this more frequently occurs with progressive or relapsed disease [16].

Diffuse non-Hodgkin's lymphomas are extremely variable and no single clinical description can adequately describe them. Small lymphocytic non-Hodgkin's lymphoma

is characterized by either focal or generalized lymphadenopathy and often splenomegaly. The disease is the tissue manifestation of the same monoclonal proliferation of the B lymphocyte that is associated with chronic lymphocytic leukemia (CLL). This disease, like CLL, most often occurs after the fifth decade and tends to be slowly progressive. The bone marrow is usually involved with this type of lymphoma, with occasional occurrences of a monoclonal gammopathy or hypogammaglobulinemia.

The most common diffuse lymphoma subtype, diffuse large B-cell lymphoma (DLBCL), often occurs as a relatively localized but rapidly progressive disease. Patients with DLBCL often present with disease in the lymph nodes; however, this subtype can occur in non-lymphatic tissues. For example, occurrences in the gastrointestinal tract, testes, thyroid, skin, breast, CNS, or bone are more frequently seen in diffuse histologies than in nodular non-Hodgkin's lymphoma [17–21]. Occurrence of non-Hodgkin's lymphoma in Waldeyer's ring is associated with an approximate 20% risk of simultaneous or subsequent involvement of the gastrointestinal tract in other sites [22].

Because of this association, when patients present with involvement in Waldeyer's ring, a complete evaluation of the small and large bowel should be included in their initial evaluation. Bone marrow involvement with DLBCL at presentation is unusual, with only 10% of the cases being positive. Diffuse infiltration of visceral organs by the aggressive diffuse lymphomas is very rare at presentation; however, relapsed or progressive lymphoma can cause these manifestations. Invasiveness of the aggressive diffuse lymphomas can result in peripheral nerve compression, spinal cord compression, great vessel or airway compression, or painful osseous lesions.

Lymphoblastic lymphoma is histologically and cytologically similar to acute lymphocytic leukemia and is most commonly seen in older children and young adults. Approximately 50% of patients present with mediastinal lymphadenopathy, and a higher percentage are males. Although the disease may appear to be localized at the time of diagnosis, there is usually rapid dissemination to the bone marrow, peripheral blood, and meninges [23]. Burkitt's non-Hodgkin's lymphoma, originally described in endemic Africa, usually presents with large extranodal tumors involving the bones of the jaw and abdominal tumors [24]. In contrast, sporadic cases in the USA most often present with abdominal tumors, with subsequent bone marrow and CNS involvement as the disease progresses [25].

Diagnosis

Because every enlarged lymph node does not necessarily represent NHL, histologic diagnosis is the most important first step toward initiating proper care of the patient. Palpable lymphadenopathy can be found in normal patients; however, the size is normally less than 1 cm. Lymphadenopathy could be associated with several benign conditions such as infectious mononucleosis, syphilis, cytomegalovirus, autoimmune disorders, granulomatous diseases, or drug hypersensitivities such as that seen with phenytoin. Since many patients with NHL are in the younger age range, infectious mononucleosis should be considered before a lymph node biopsy. A lymph node biopsy specimen from this condition is sometimes difficult to distinguish from NHL [26]. Patients with lymphomatoid granulomatosis have a 12% incidence of developing overt lymphoma [27]. Many other malignant conditions such as metastatic carcinomas, germ cell neoplasms, or melanomas can also present with lymphadenopathy. Young male patients with malignant axillary lymph node enlargement are most likely to have a lymphoma or a malignant melanoma. In women, the same two tumors as well as breast carcinoma would be the most likely diagnosis.

An adequate biopsy is essential for diagnosis, and an excisional biopsy should be performed. The largest or most rapidly enlarging node may be chosen because it may reveal the most aggressive histology and provide the most tissue. Fine-needle aspiration is usually not adequate for initial diagnosis but may be used to confirm relapse. Every patient with NHL does not always present with peripheral lymphadenopathy that is easily biopsied, and other procedures may be required for diagnosis, but must be approached with caution. Other sites of presentation that may require biopsy for diagnosis include skin, gastrointestinal tract, breast, or testes. Visceral presentation such as hepatic, splenic, or pulmonary involvement may occasionally require an open biopsy for diagnosis. Also, retroperitoneal, mesenteric, or pelvic disease may require a diagnostic laparotomy for diagnosis confirmation. Unexplained neutropenia, anemia, a leukoerythroblastic picture, or thrombocytopenia necessitates a bone marrow biopsy that usually diagnoses marrow involvement with NHL. Patients at risk of CNS involvement should undergo lumbar puncture with evaluation of cerebrospinal fluid by cytology and flow cytometry [28]. Burkitt's lymphoma and extranodal DLBCL are associated with increased risk of CNS disease and should also undergo lumbar puncture during initial diagnostic testing [29]. Furthermore, many treatment regimens contain chemotherapy CNS prophylaxis for these high-grade lymphomas with increased risk of CNS disease. The storage of fresh-frozen or paraffin embedded tissue is highly encouraged for future diagnostic tests based on molecular profiling.

When a lymphoma is suspected clinically, the pathologist should be notified so that special processing procedures can be used if they are available, including cytogenetics, surface markers, immunohistochemistry stains, and molecular biologic studies, in addition to routine histology. These

additional studies can add valuable information to the cases that are difficult to identify on routine testing and provide insight into the aggressiveness of the subtype. Patients with an undifferentiated neoplasm by routine histology can have a NHL in a small percentage of cases. Because this is one of the more treatable causes of what appears to be an undifferentiated neoplasm, special stains should be an important part of the evaluation of a specimen with this initial diagnosis. The accuracy and reproducibility of pathologists in the diagnosis of NHL are essential for the care and treatment of patients. Even the expert pathologists who developed the Working Formulation were able to reproduce their initial diagnosis on re-reviewing a slide only 53–93% of the time [30].

Evaluation and Staging Procedures

A thorough history and a physical examination are important for optimal management. Patients should be questioned about systemic symptoms, and performance status should be assessed. Further questions should be directed to potential causative factors, such as prior malignancy, chemotherapy, or radiation treatment, autoimmune or immunodeficiency diseases. This includes a detailed history for exposure to HIV, hepatitis C, and HTLV-1. No NHL patients should be managed without a comprehensive physical examination, with detailed attention to lymph nodes, liver, and spleen (Table 45.2).

Once a histologic diagnosis of NHL has been confirmed by biopsy, various staging procedures must be done to direct the therapy as well as to provide for prognostic information for the patient. The histologic subtype and the condition of the patient direct the type and speed of staging procedures. For example, a patient with an aggressive NHL that is rapidly progressive requires prompt staging procedures that are the minimum required before the initiation of therapy. On the other hand, indolent non-Hodgkin's lymphoma can be staged at the convenience of the patient and physician. Treatment decisions can then be made with all the knowledge from the staging procedures available for discussion.

Baseline studies that should be obtained in most patients include complete history and physical examination; blood testing including complete blood count, platelet count, liver chemistries, lactate dehydrogenase (LDH), calcium, and renal function studies; computed tomography (CT) of the chest, abdomen, pelvis; PET scan; and bone marrow biopsy. Additional studies that may be appropriate in certain circumstances include cytologic examination of any effusion present (i.e., pleural or pericardial effusions or ascitic fluid) and viral serologies for hepatitis C, HIV, or HTLV-1. EBV viral loads can be useful in nasal NK/T-cell lymphomas or post-transplant lymphoproliferative disorders (PTLD) [31, 32]. A thorough evaluation of the gastrointestinal tract should be pursued if any symptoms are present or if the primary presentation of lymphoma was in Waldeyer's ring.

The clinical presentation and sites of disease usually dictate the need for further diagnostic testing. CT or magnetic resonance imaging (MRI) of the brain or spinal cord should be performed if any signs or symptoms of CNS lymphoma or high-grade lymphomas such as Burkitt's lymphoma or extranodal DLBCL are present. 18-Fluoro-2-deoxyglucose (FDG)-positron emission tomography (PET) provides sensitive functional imaging for lymphomas for initial assessment and end of treatment response. For example, 98–100% of follicular, mantle cell, and DLBCL NHL are detectable by

Table 45.2 Staging evaluation of lymphomas

Evaluation	Mandatory	As indicated
Confirm diagnosis	Adequate biopsy reviewed by experienced hematopathologists	Immunophenotyping
		Cytogenesis
		Molecular studies
General overview	Careful history and physical examination	Blood coagulation studies
	Complete blood count (including platelet count)	Serum and viral protein studies
	Chemistry screen (including liver and renal function studies)	Serum electrolytes, uric acid, Hepatitis B testing
Prognostic categorization	Serum LDH	Erythrocyte sedimentation rate
	Serum albumin	Serum β_2-microglobulin
		Tumor growth fraction
		Microarray analysis
Anatomic disease	Whole-body CT scans	Ultrasonography
		Magnetic resonance imaging
		FDG-PET scans
		Upper/lower GI scope
Occult sites of involvement	Bone marrow biopsy	Lumbar puncture with flow cytometry
		Biopsy of suspicious sites
		Blood flow cytometry
		Blood and bone marrow PCR

FDG-PET [33]. Although a few studies has shown the prognostic value of FDG-PET to predict early relapse, there does not appear to be convincing literature to support the use of surveillance imaging, including FDG-PET, CT, or MRI [34]. After therapy has been initiated, and at appropriate intervals, previously positive tests should be repeated for evaluation of the effectiveness of the therapy.

The results of these tests are utilized for designation of the proper staging classification for the patient. Patients are classified according to the Ann Arbor staging system, which was originally developed for Hodgkin's lymphoma [35]. The histologic subtype, presence or absence of B symptoms, and the classical Ann Arbor stage are important for initial evaluation and stratification of the patient for appropriate treatment. However, the heterogeneity and hematogenous pattern of dissemination in NHL causes the staging system to have more limited value when compared to the contiguous lymphoma node spread with Hodgkin's lymphoma. Consequently, important modifications to the Ann Arbor staging system were made at the Cotswold Conference, thereby making it more applicable to NHL [36]. The Cotswold modification includes a framework to measure the number of anatomic sites, the "bulk" of the disease, and the type or number of extranodal sites (Table 45.3).

Table 45.3 Cotswold staging of lymphomas

Stage	Features
I	Involvement of a single lymph node region or lymphoid structure (e.g., spleen, thymus, and Waldeyer's ring)
II	Involvement of two or more lymph node regions on the same side of the diaphragm
III	Involvement of lymph regions or structures on both sides of the diaphragm
IV	Involvement of extranodal site(s) beyond that designated E
For all stages	
A	No symptoms
B	Fever (>38 °C), drenching sweats, and weight loss (10% body weight over 6 months)
For stages I–III	
E	Involvement of a single extranodal site contiguous or proximal to known nodal site
Cotswold modifications	
(i)	Suffix to designate bulky disease as more than one-third widening of the mediastinum or >10 cm maximum dimension of nodal mass
(ii)	The number of anatomic regions involved should be indicated by a subscript (e.g., II3)
(iii)	Stage III may be subdivided into:
	III1, with or without splenic, hilar, celiac, or portal nodes
	III2, with para-aortic, iliac, or mesenteric nodes
(iv)	Staging should be identified as clinical stage or pathologic stage
(v)	A new category of response to therapy, unconfirmed/uncertain complete remission should be introduced because of the persistent radiologic abnormalities of uncertain significance

Prognostic Factors

Histopathology

Many histologic classifications have been formulated, in an attempt to classify the NHL into prognostic categories [37–40]. Histologic classification schemes have been developed to organize lymphomas into groups with shared pathogenesis and clinical behavior with an aim to guide treatment. The Kiel classification (accepted in Europe) incorporated information of the cell of origin, while the Working Formulation (accepted in North America) provided clinical grouping and pathologic divisions that were largely based on cell size (small vs. large), cell shape (round vs. not round), and growth pattern (follicular vs. diffuse) [38, 39]. The Working Formulation was a further attempt to create an easily applied classification that would help to improve the prognostic significance of the histologic diagnosis, which included low-, intermediate-, and high-grade categories [39]. Over the years, these classifications have evolved from exclusively morphologic classifications to those that incorporate immunophenotype and molecular genetic information. The Revised European-American Classification of Lymphoid Neoplasms (REAL) offered a histopathologic classification containing some entities felt to represent specific clinical-pathologic cases that may behave in a specific clinical manner [40]. The evolution of the various classification systems is the result of how the molecular genetic information has been applied to understand the molecular pathogenesis of lymphoma, by way of the identification of hallmark genetic abnormalities. This has ultimately culminated in the World Health Organization Classification of Neoplastic Diseases of Hematopoietic and Lymphoid Tissues [40], which is now the most widely accepted classification system (Table 45.4). A revised 4th edition without any new definite entities was released in 2016. This update attempts to incorporate new diagnostic, prognostic, and therapeutic implications of research over the 8-year gap since the last WHO classification was released [41].

Histologic transformation from a low-grade to a high-grade NHL is associated with more aggressive behavior of the lymphoma and a poor prognosis. Histologic transformation occurs in approximately 5–40% of follicular or small lymphocytic lymphomas, usually many years after the original diagnosis [42, 43]. The transformation of small lymphocytic lymphoma to DLBCL was originally described many years ago and given the name Richter's syndrome [44]. This transformation occurs in approximately 30% of these patients; however, a larger proportion of patients undergo a gradual acceleration in the clinical course of their disease over time. In general, DLBCL comprises multiple disease entities as suggested by its variable clinical presentation, natural history, morphologic variants, and molecular

Table 45.4 WHO classification of lymphomas

Mature B-cell neoplasms
• Chronic lymphocytic leukemia/small lymphocytic lymphoma
• B-cell prolymphocytic leukemia
• Lymphoplasmacytic lymphoma (such as Waldenström's macroglobulinemia)
• Splenic marginal zone lymphoma
• Hairy cell leukemia
• Plasma cell neoplasms
– Plasma cell myeloma
– Plasmacytoma
– Monoclonal immunoglobulin deposition diseases
– Heavy chain diseases
• Extranodal marginal zone B-cell lymphoma, also called MALT lymphoma
• Nodal marginal zone B-cell lymphoma (NMZL)
• Follicular lymphoma
• Primary cutaneous follicle center lymphoma
• Mantle cell lymphoma
• Diffuse large B-cell lymphoma, NOS
– Germinal center B-cell type
– Activated B-cell type
• Primary mediastinal (thymic) large B-cell lymphoma
• Primary DLBCL of the central nervous system
• Intravascular large B-cell lymphoma
• Primary cutaneous DLBCL, leg type
• ALK+ large B-cell lymphoma
• Plasmablastic lymphoma
• Intravascular large B-cell lymphoma
• Primary effusion lymphoma
• Burkitt's lymphoma
• B-cell lymphoma, unclassifiable, with features intermediate between DLBCL and classical Hodgkin lymphoma
• Posttransplant lymphoproliferative disorders (PTLD)
Mature T-cell and natural killer (NK) cell neoplasms
• T-cell prolymphocytic leukemia
• T-cell large granular lymphocytic leukemia
• Aggressive NK cell leukemia
• Adult T-cell leukemia/lymphoma
• Extranodal NK/T-cell lymphoma, nasal type
• Enteropathy-type T-cell lymphoma
• Hepatosplenic T-cell lymphoma
• Subcutaneous panniculitis-like T-cell lymphoma
• Blastic NK cell lymphoma
• Mycosis fungoides/Sezary syndrome
• Primary cutaneous CD30-positive T-cell lymphoproliferative disorders
– Primary cutaneous anaplastic large cell lymphoma
– Lymphomatoid papulosis
– Primary cutaneous gamma-delta T-cell lymphoma
– Primary cutaneous CD8+ aggressive epidermotropic cytotoxic T-cell lymphoma
• Angioimmunoblastic T-cell lymphoma
• Follicular T-cell lymphoma
• Peripheral T-cell lymphoma, NOS
• Anaplastic large cell lymphoma (ALK+, ALK-)

characteristics [45]. The diverse morphologic variants include germinal center B-cell type, activated B-cell type, and EBV+ DLBCL, NOS have some known molecular correlates and are beginning to be recognized as distinct diseases. Other DLBCL subtypes such as primary mediastinal B-cell lymphoma (PMBL), primary cutaneous leg-type, T-cell/histiocyte-rich, primary DLBCL of the central nervous system, and intravascular large B-cell lymphoma have been identified as distinct subtypes of DLBCL for longer based on a combination of clinical, histologic, and molecular findings [40]. It is also important to recognize that large cell "transformation" of an indolent B-cell lymphoma/leukemia, a relatively common occurrence, involves pathways of lymphomagenesis that are distinct from those of de novo DLBCL, but share similar histologic, immunophenotypic, and oncogenetic characteristics [45].

Sites of Disease

Most NHL occur within lymph nodes; however, several extranodal presentations are seen with some frequency. Primary CNS lymphomas were previously rarely reported. However, with the acquired immunodeficiency syndrome (AIDS) epidemic, many more primary CNS lymphomas have been noted [46]. Most non-AIDS-associated primary CNS lymphomas are small non-cleaved lymphoblastic, and DLBCL lymphomas. Many of the series in the literature are older and involve the delivery of less than optimal therapy. However, with newer techniques, approximately one-fourth of the patients have prolonged disease-free survival [47, 48]. Patients with the AIDS-associated primary CNS NHL have had a much poorer median survival of 3 months [49]. Another extranodal area that may be involved with NHL is the testes. The histology is most frequently DLBCL and most patients are elderly. In most previous series, patients treated with orchiectomy alone relapsed with disseminated disease in a few months [50]. More recent series have improved results utilizing combined modality of chemotherapy including intrathecal prophylaxis and contralateral testes radiation [51]. Considering the gastrointestinal tract as a whole, primary lymphoma of the gastrointestinal tract is the most common extranodal site. As noted previously, involvement of Waldeyer's ring correlates with an increased risk of gastrointestinal lymphoma up to a 20% incidence. The most frequent gastrointestinal location for lymphoma is gastric, followed by small intestine, ileocecal area, and large intestine [52]. NHL of the gastrointestinal tract has been associated with nontropical sprue [53] and systemic sclerosis [54]. Because of their location, some gastrointestinal lymphomas must be treated with local surgical excision before initiation of chemotherapy or radiation therapy, especially extensive small or large intestine lesions. Gastric lymphomas can occasionally be treated with chemotherapy without the necessity of prior gastrectomy.

Age

Age has been found to be a prognostic factor in several studies [55]; however, several other studies have not confirmed this fact. When age groups have been stratified for causes of death other than lymphoma, one study found no difference in the adjusted survival of older patients [56]. However, older patients often have more difficulty with chemotherapy side effects and may have increased morbidity due to drug-related toxicity [57]. Several studies report a diminished complete response rate in the elderly patients; however, it is not clear whether this is due to lower drug doses administered or other factors [58]. In most large unselected series, the median age of the patients is usually in the early to mid-sixties. However, patients in many chemotherapeutic trials have a median age of 45–55 years. With this age difference in clinical trials, comparisons are difficult between the various aggressive regimens. Further evaluation of these regimens in unselected patient populations may lead to different results in older patient populations.

Tumor Bulk

Several studies have now identified larger tumor bulk as an adverse prognostic factor [59]. Tumor bulk is defined in different studies from 5 to 10 cm. The complete remission rate and the relapse rate have been found to be inferior in patients with large tumor bulk. In patients with stage I and II disease who were treated with radiation therapy only, one study found that those with a tumor size less than 3 cm in diameter had a 5% local recurrence rate compared with an 18% local recurrence rate with tumors 3 cm or more in diameter [60].

Laboratory Analysis

Elevated serum LDH has also been associated with a poor prognosis in a number of analyses [61]. A number of analyses performed on the lymphoma tissue itself have recently been shown to be helpful in predicting outcome with therapy. The immunophenotype is now fundamental for the accurate classification of lymphoma, with most of B-cell origin. Immunophenotyping studies have shown in a number of studies that a T-cell immunophenotype is an adverse prognostic factor [62, 63], although a few other studies have not confirmed this fact [64].

Indicators of rapid proliferation such as Ki-67 and DNA proliferative index have also been analyzed for their relationship to prognosis. Investigators at Arizona evaluated 105 patients with DLBCL utilizing the anti-Ki-67 monoclonal antibody. In their analysis, 19 patients with Ki-67 levels higher than 60% had a median survival of 8 months compared with a median survival of 39 months for the 86 patients with Ki-67 levels of 60% or less [65]. Proliferative activity and DNA aneuploidy as identified by flow cytometry have also been evaluated for use as predictive indicators of prognosis in patients with DLBCL. One study of 50 newly diagnosed patients found that high proliferative activity was the single most important pretreatment adverse prognostic factor in these patients. In that study, DNA aneuploidy was detected in 62% of cases; however, it did not seem to have any prognostic significance.

Molecular genetics have advanced the understanding of lymphomagenesis by the identification of relevant genes that are important in proliferation, differentiation, and death. The application of this technology has further provided vital insights into the unique molecular signatures of distinct types of B-cell malignancies, thereby relating lymphomas to normal stages in B-cell development and physiology [66, 67]. The molecular consequences include oncogene activation or loss of tumor suppressor genes caused by a variety of chromosomal lesions, including translocation, deletion, or mutation. Most of these genetic lesions are currently associative, since the downstream effect of these abnormalities is not well understood. Regardless, this molecular understanding has provided new ways to classify and predict clinical outcome, which is certainly the pathway to targeted and individualized therapy for NHL.

The identification of nonrandom chromosomal abnormalities in patients with NHL has been described in multiple studies [68–71]. Certain chromosomal abnormalities can be associated with specific histologic subtypes of lymphoma such as t(14;18)(q32;q21) in follicular lymphomas [70], t(11;14)(q13;q32) in mantle cell lymphoma (MCL) [71], and t(8;14)(q24;q32) in Burkitt's lymphoma [69]; structural abnormalities such as the deletion of 6q can be associated with NHL as well [72]. Other chromosomal changes are outlined in Table 45.5. The genetic hallmark of follicular lymphoma, t(14;18)(q32;q21), leads to the rearrangement and overexpression of the bcl-2 gene and is present in up to 90% of cases. Inhibition of apoptosis by bcl-2 plays an important role in lymphomagenesis [72]. Interestingly, bcl-2 overexpression is turned off in follicular lymphoma cells that have undergone aggressive transformation.

The common molecular abnormality of DLBCL involves deregulation of bcl-6, with 35% of cases illustrating an abnormality in the 3q27 region [73]. Bcl-6 is a transcription factor that is important in the normal functioning of the germinal center B cells as well as the germinal center cell formation during antigen presentation [74]. Therefore, DLBCL with high expression of bcl-6 are usually of germinal center B-cell origin, which distinguishes them from other DLBCL

Table 45.5 Molecular abnormalities in lymphomas

NHL histologic type	Translocation	Proto-oncogene involved	Proto-oncogene function
Lymphoplasmacytic lymphoma	t(9 114)(p13;q32)	*pax-5*	Transcription factor regulating B-cell proliferation and differentiation
Follicular lymphoma	t(14;18)(q32;q21)	*bcl-2*	Negative regulator of apoptosis
	t(2;18)(p11;q21)		
	t(18;22)(q21;q11)		
Mantle cell lymphoma	t(11;14)(13;q32)	*bcl-1/cyclin D1*	Cell cycle regulator
MALT lymphoma	t(11;18)(q21;q21)	*api_2/mlt*	*api_2* has anti-apoptotic activity
	t(1;14)(p22;q32)	*bcl-10*	Anti-apoptosis
Diffuse large B-cell lymphoma	del(3)(q27)	*bcl-6*	Transcriptional repressor required for GC formation
Burkitt's lymphoma	t(8;14)(q24;q32)	*c-myc*	Transcription factor regulating cell proliferation and growth
	t(2;8)(p11;q24)		
	t(8;22)(q24;q11)		
Anaplastic large T-cell lymphoma	t(2;5)(p23;q35)	*npm/alk*	*alk* is a tyrosine kinase

subtypes [75]. The exact functional significance is largely unknown, although some studies have found an improved survival in these patients [76].

Burkitt's lymphoma is characterized by a translocation between *c*-myc on chromosome 8 and one of the three immunoglobulin chain loci, occurring with a respective frequency of 80%, 15%, and 5% on chromosomes 14, 22, and 2, respectively, which encode in turn for the IGH and lambda and kappa light chains [40]. t(8:22) is usually found in lambda-expressing cells, whereas the t(8:2) is found in kappa-expressing cells. The quantitative overexpression of c-myc has been shown to result in deregulation of cellular growth and is capable of blocking phenotypic maturation. Therefore, c-myc overexpression appears to be central to the pathogenesis of BL, and it is thought that the translocation occurs in pre-B-cell development during the normal rearrangement of the immunoglobulin loci. MCL cases almost always contain the t(11;14)(q13;q32) translocation between the IGH and the cyclin D1 (PRAD1, bcl-1) genes [77]. Deregulation of bcl-1 leads to overexpression of its gene product cyclin-D1, which promotes progression from G1 to S of the cell cycle [78].

International Prognostic Indices

Clinical stage is a powerful and independent predictor of prognosis. The largest evaluation of prognostic factors for aggressive lymphoma was made in 2031 patients and published as the International Prognostic Index [79]. In the multivariate analysis, age (less than 60 years vs. 60 years or older), tumor stage (I–II vs. III–IV), serum lactate dehydrogenase (normal vs. higher than normal), performance status (ECOG 0–1 vs. 2 or higher), and number of extranodal sites of disease (0 or 1 vs. 2 or more) were identified as being highly predictive of outcome. These risk factors were put together into an analysis so that the number of factors was highly predictive of outcome. Patients with 0 or 1 risk factor had a complete remission (CR) rate of 87% and a 5-year survival rate of 73%; those with two risk factors had a CR rate of 67% and a 5-year survival rate of 51%; with three risk factors, the rate was 55% and the 5-year survival rate was 43%; with four or five risk factors, the CR rate was 44% and the 5-year survival rate was 26%. This prognostic index should be evaluated in all new patients with aggressive lymphoma to plan their clinical courses, since it is the standard for assessing clinical prognosis and treatment stratification. Although it has yet to be fully revalidated in the rituximab era, a revised prognostic model for R-CHOP (Revised-IPI) is available from retrospective analysis [80]. Other prognostic indices have evolved for other subtypes of lymphoma. The Follicular Lymphoma International Prognostic Index (FLIPI) has been validated, as age, stage, LDH, hemoglobin level, and number of nodal areas reliably predict survival [81]. Finally, a validated prognostic index for MCL (MIPI) has been published and also widely accepted [82, 83] (Table 45.6).

Gene expression profiling has emerged as an important prognostic tool. On the basis of gene signatures, DLBCL can be divided into at least three different subtypes: germinal center B-cell-like (GCB), activated B-cell-like (ABC), and primary mediastinal B-cell lymphoma (PMBC). The term non-GCB is reserved for classification based on immunohistochemistry protein expression rather than gene expression. These categories possess a different overall survival and superiority in patients with the germinal center type versus the activated type [68]. Using these gene expressions profiled, a molecular prognostic model of survival has been developed for CHOP-treated DLBCL and is independent of IPI. However, like many new facets of NHL treatment, this molecular prognostic model has yet to be validated in the rituximab era. Gene expression

Table 45.6 Prognostic classification systems for lymphomas

International Prognostic Index for aggressive lymphomas			
Risk group	IPI score[a]	CR rate (%)	5-Year OS rate (%)
Low	0, 1	87	73
Low intermediate	2	67	51
High intermediate	3	55	43
High	4, 5	44	26
Revised International Prognostic Index (R-IPI)			
Risk group	IPI score[a]	4-Year OS rate (%)	
Very good	0	94	
Good	1–2	79	
Poor	3–5	55	
Follicular International Prognostic Index (FLIPI)			
Risk group	IPI score[a]	Distribution (%)	10-Year survival rate (%)
Low	0–1	36	71
Intermediate	2	37	51
Poor	≥3	27	36
Mantle Cell International Prognostic Index (MIPI)			
Risk group	MIPI score = [0.03535 × age (years)] + 0.6978 (if ECOG > 1) + [1.367 × log10 (LDH/ULN)] + [0.9393 × log10 (WBCs per 10^{-6} L)]	Distribution (%)	5-Year survival rate (%)
Low		80	82
Intermediate		41	75
High		37	35

CR complete response, *ECOG* Eastern Cooperative Oncology Group, *IPI* International Prognostic Index, *LDH* lactate dehydrogenase, *OS* overall survival

[a]One point is given for the presence of each of the following characteristics: age > 60 years, elevated serum LDH level, ECOG performance status ≥2, Ann Arbor stage III or IV, and more than two extranodal sites

profiling for other types of lymphoma have also found subsets with different prognostic outcomes. For example, in follicular lymphoma, two subsets were found based on the immune effector cells in the microenvironment of the involved lymph nodes and in an 81 gene expression panel [84, 85].

Treatment

Background

Optimized care requires an understanding of a comprehensive approach to all medical facets of each patient, and most importantly, the correct histologic diagnosis. The treatment strategy for NHL varies based on several factors including histologic subtype, stage, age, performance status, and other prognostic factors, as discussed under patient staging and evaluation. However, it is important to be mindful that the WHO classification is not organized with treatment in mind. Management of acute problems such as spinal cord compression, superior vena cava syndrome, hypercalcemia, and obstruction may specifically direct initial therapy. Adequate treatment is often multidisciplinary, involving surgical oncologists, medical oncologists, and radiation oncologists. The initial goal of therapy is generally the attainment of a complete remission defined as the documented disappearance of disease from previously involved sites. In general, treatment response should be documented by physical findings, and all abnormal tests should be repeated. The guidelines outlined by a workshop of the National Cancer Institute in 1998 and more recently in 2007 and 2013 should be used to follow response, as this workshop standardized and refined initial evaluation and response criteria [86–88]. Final treatment assessment is usually performed 4–6 weeks after completion of therapy unless progression occurs earlier. The frequency and extent of investigations during follow-up depend on the risk of recurrence. Although a common practice, routine surveillance radiographic analysis has not proven to be predictive of outcomes in studies. Further discussion of treatment has been organized based on shared biologic and clinical features.

The fundamental genetic changes that lead to lymphoma involve deregulation of cellular proliferation and death, events that are controlled by cell cycle checkpoints and by the induction or suppression of apoptosis. Abnormalities in these pathways might determine the sensitivity of tumor cells to the cytotoxic effects of irradiation and chemotherapy [89, 90]. Clinical strategies which take advantage of these abnormalities in the regulation of the cell cycle and

apoptosis have an increasingly important role in the design of novel therapeutic approaches. The classic principles of chemotherapy, including pharmacology, drug resistance, and tumor cell kinetics, continue to form the foundation of treatment strategies. Understanding these principles provides the basis for good treatment decisions. Dose intensity has long been considered important in the treatment of NHL; however, the heterogeneous nature of NHL and the addition of rituximab to most treatment schema have diminished differences in response to more toxic chemotherapy regimens.

Indolent Histologic Subtypes

Follicular Lymphoma

The so-called favorable indolent histologic subtypes of cutaneous T-cell lymphoma (CTCL), and the small lymphocytic (SL), follicular small cleaved (FSC), and follicular non-Hodgkin's lymphoma are not as common as the aggressive NHL's. The indolent histologies compose 20–45% of the lymphomas in most series. While follicular NHL can occur in younger patients, CTCL and SL lymphoma most often occur in patients over 60 years of age. When careful staging procedures as outlined previously are followed, over 90% of the patients have advanced stage disease at diagnosis.

Patients with these indolent histologic subtypes have a prolonged natural history with a median survival in most series of 10–15 years or more [91, 92]. Also, except in a few cases of truly localized disease, these indolent histologic subtypes of NHL cannot be cured. The therapy offered to the patient must take this incurability into account. Patients with few serious signs or symptoms of their disease can be placed into the "watch and wait" category until they show signs or symptoms of their disease, making treatment essential [93, 94].

Treatment of these NHL is generally initiated for constitutional symptoms, organ compromise, and/or evidence of rapid tumor proliferation. Most patients under these circumstances have bulky disease, diffuse bone marrow infiltration, and threatened organ function. When the disease does advance so that treatment is necessary, localized radiation therapy can be used for symptomatic adenopathy; also, single or combination chemotherapeutic agents can be used for more extensive disease. Multiple chemotherapeutic agents have been found to be effective in this clinical situation [95–98], including purine analogs, alkylators, anthracyclines, and rituximab. In addition, targeted agents, radioimmunotherapy, and allogeneic stem cell transplantation are now in current treatment paradigms. With most of these modalities, a complete or partial response can be achieved at least initially. However, a relapse rate of 10–15% per year occurs, with only a small percentage of patients remaining in remission after 10 years of follow-up.

Table 45.7 lists common chemotherapy regimens for indolent NHL [99–104].

Follicular lymphomas are classified into three grades based on the number of centroblasts per high-power field; grades 1 and 2 are indolent, whereas grade 3 is more aggressive. Grade 3 disease is biologically diverse and includes both grade 3a follicular lymphoma and grade 3b large B-cell lymphoma, which has a follicular growth pattern but clinically resembles DLBCL [105]. A number of clinical features are associated with outcome of follicular lymphoma (FL). In a study of 4000 patients with FL, age greater than 60 years, Ann Arbor stage III or IV, hemoglobin level less than 12 g/dL, abnormal serum LDH level, and involvement of five or more lymph node areas were adverse prognostic features, which are the criteria that make the FLIPI more discriminating than the IPI.

Most follicular lymphomas present at advanced stage, but approximately 15–30% are stage I or II. Based on retrospective studies, radiotherapy alone has been the standard of care, with median survival approaching 15 years and up to one-third of patients being progression free. It is difficult to determine if these results reflect the benefit of radiotherapy or the indolent nature of early stage disease. A retrospective

Table 45.7 Common chemotherapy regimens for indolent NHL

Clinical group	Treatment	Complete response (%)	Overall response (%)	Median PFS range
Untreated	CVP ± R	10–40	60–80	3–5 Years
Symptomatic	CHOP ± R	40–80	90–100	5–7 Years
	FND ± R	70–90	90–100	5–7 Years
	R ± Maintenance R	15–40	60–75	2–3 Years
	Bendamustine +R	30–40	90–100	5–6 Years
Relapsed	Rituximab	10–20	40–60	8–24 Months
	Radioimmunotherapy	15–50	65–80	8–24 Months
	Chemotherapy	15–40	60–90	12–24 Months
	Idelalisib	6	57	11 Months

CHOP cyclophosphamide, adriamycin, vincristine, and prednisone, *CVP* cyclophosphamide, vincristine, and prednisone, *FND* fludarabine, mitoxantrone, and dexamethasone, *OS* overall survival, *PFS* progression-free survival, *R* rituximab

analysis of 43 patients with untreated stage I or II FL found that 63% did not require therapy over the median follow-up of 86 months and only four patients showed histologic transformation, with a survival rate of 85% [106]. Combination chemotherapy followed by involved-field radiation therapy for early stage disease yielded a similar 10-year survival rate of 82% [107]; however, the benefit of treatment was further negated by two cases of myelodysplasia and 12 secondary malignancies. Although it can cause long-term toxicities, radiotherapy still remains a popular treatment of choice [108]. Quite simply, these data illustrate that no clear perfect treatment exists, other than a treatment strategy that is individualized for each patient.

Several randomized trials have compared observation with single-agent or combination chemotherapy in patients with asymptomatic advanced stage lymphoma. Aggressive treatment with ProMace-MOPP was compared with observation, but this study did not prove that early treatment was beneficial [93]. Another randomized study compared observation to chlorambucil, and also demonstrated a similar OS at a follow-up of 16 years [109]. Although these two randomized trials support observation, they were performed before the use of rituximab, which has been shown to prolong survival in symptomatic patients [110]. For asymptomatic patients, single agent rituximab induction and 2 year maintenance was compared with a watch and wait approach and showed 86% versus 46% of patients did not need new treatment at 3 years. Giving only 1 month of rituximab weekly was no worse than 2 years in terms of needing new treatment at 3 years. There was no improvement in quality of life with therapy although illness coping and mental adjustment to cancer scores were better in the treatment groups [111].

Initial chemotherapy for patients with symptomatic disease should be tailored based on the nature and pathobiology of the disease; for example, high tumor burden usually requires combination regimens, while slower progressive disease may require a single agent regimen. Table 45.7 contains a representation of the common regimens employed.

Perhaps the most important advance in therapy for indolent lymphomas has been the use of rituximab (chimeric anti-CD20). In the original trials, rituximab was administered weekly for 4 weeks to patients with multiple relapsed indolent NHL. They found a CR rate of 6% and an overall response rate of 50% in the trial [101]. It has also been used in combination with CHOP in patients with indolent NHL and found to have a 97% response rate [100]. This antibody has evolved into the standard therapy for many patients with indolent NHL.

Other trials have shown statistically significant response rates, progression-free survival (PFS), and molecular remissions [103, 112]. Rituximab was also tested as a first-line treatment, followed by maintenance therapy every 6 months in patients without progression [113]. The advantage was clearly demonstrated by a response rate of 47% following induction, with an increase to 73% during maintenance, including a 37% CR rate, and a median PFS was 34 months. The role of a 4-week induction course followed by maintenance at 3, 5, 7, and 9 months was tested in untreated or relapsed/refractory FL. The overall response rate was 67% and 46% in untreated and relapsed/refractory patients, respectively, and the median EFS was 12 and 23 months for the observation and maintenance groups, respectively [105]. Untreated patients benefited most from maintenance, with an EFS increasing from 19 to 36 months. Additionally, the large multinational PRIMA trial gave half of the subjects maintenance rituximab every 8 weeks for 2 years after rituximab plus chemotherapy. This resulted in a 75% PFS versus 58% in the no maintenance group, although overall survival did not differ [114].

Without question, rituximab has improved response and survival when added to standard conventional chemotherapy regimens. R-CHOP is clearly superior to CHOP on the basis of overall response rates, median PFS, and a slight survival advantage [115]. When CVP was compared with and without rituximab, the addition of rituximab also significantly improved response, progression, and survival [102]. Other combinations where rituximab has been used include F-RCM and R-FND regimens, as well as after FM and CHOP in patients who did not achieve a molecular remission [103]. These trials clearly indicate that rituximab significantly improves the efficacy of chemotherapy and OS.

Other promising new combinations with rituximab include bendamustine (BR) and lenalidomide (R^2). The BR regimen showed impressive PFS in the first line when compared directed to R-CHOP in a German study of indolent lymphomas. With a median follow-up of 45 months, median PFS was not reached versus 40.9 months with a hazard ratio of 0.61 for BR [98]. These data were supported by the BRIGHT study which showed BR approached non-inferiority to R-CHOP for a complete response (CR-rate ratio 1.27 p = 0.0569) with a trade-off of more nausea and drug-hypersensitivity reactions [116]. Data for PFS and OS were not yet mature. The use of lenalidomide with rituximab has shown in a small phase II trial to give an 87% complete response and an additional 11% partial response [117]. In the second line, overall response rate was 76% with a median time to progression of 2 years [118]. An international phase 3 study with R^2 is currently in progress.

Patients who show benefit from an initial treatment can be retreated in a relapsed setting with the same agents, although alternative combinations may be selected. Idelalisib is a first in its class oral inhibitor of the delta isoform of the phosphatidylinositol 3-kinase located in the lipid bilayer of B cells. In a phase 2 study of heavily pretreated patients with indolent lymphomas, response rates were 57% with a median duration of response of 12.5 months [104].

Anti-CD20 antibody therapy has been combined with radioactive isotopes. There were two drugs commercially available in the form of iodine-131 tositumomab (Bexxar) and yttrium-90 ibritumomab tiuxetan (Zevalin); however, Bexxar was discontinued in the US in February 2014. There was a lack of comparative single agent studies and restrictions of administration led to a low usage rate and ultimate discontinuation of an effective drug from the market. Ibritumomab tiuxetan showed a response rate of 74% in rituximab refractory patients with follicular lymphoma with time to progression of 6.8 months [119]. In the relapsed or refractory setting, Ibritumomab tiuxetan out-performed rituximab with an overall response rate of 80% versus 56% with double complete response rates but no difference in time to progression [120]. Thus the role of radioimmunotherapy is currently limited to relapsed patients with limited bone marrow involvement with adequate normal marrow elements and no evidence of MDS markers by FISH.

High-dose therapy with autologous hematopoietic stem cell support for indolent lymphoma has been tested as consolidation in first response, relapse, and transformation [121, 122]. For patients with relapsed FL, autologous hematopoietic cell transplant (Au-HCT) does appear to produce long-term disease-free survival in selected patients. The patients who appear to benefit the most are those who are transplanted after receiving <3 prior chemotherapy regimens and have a lower FLIPI at the time of relapse. For multiply relapsed FL patients, Au-HCT does not produce a favorable risk-benefit ratio [123]. However, for FL patients in first remission, the incidence of secondary malignancies, especially MDS and AML, complicates Au-HCT and must be taken into consideration for those patients with long expected lifespans. Despite recent advances in novel B-cell inhibitors, if clinical trials are not available, most centers utilize Au-HCT for selected first or second relapsed FL patients who have a shortened disease-free interval or other indications of a poor outcome with standard therapy.

Allogeneic transplantation (Al-HCT) can be performed after either conventional myeloablative or reduced-intensity conditioning [124]. The latter approach is an encouraging development since it has substantially reduced treatment-related morbidity and mortality, consisting mainly of acute and chronic GVHD and infectious complications, without a major increase in relapse risk [125]. Although relapse and progression occur less often after myeloablative allogeneic HCT, this modality should be used cautiously because of its prohibitive non-relapse mortality. Most currently published trials were initiated in the pre-rituximab era. As such, the role of Au-HCT and Al-HCT need to be re-evaluated in the setting of initial therapy with rituximab-containing regimens and prospective studies are urgently needed to validate these strategies. Individual patient evaluation and treatment planning should be considered.

Small Lymphocytic Lymphoma (SLL/CLL)

Small lymphocytic lymphoma is clinically similar to CLL. Since the typical patient population is generally older and the disease can often be indolent and asymptomatic for many years, conservative therapy is usually favored. Historically, purine analogs, such as fludarabine, cladribine, and pentostatin, alone or in combination have been employed with the best success [126]. The efficacy of fludarabine or cladribine alone has been consistently demonstrated to show response rates between 30 and 50% [127]. Fludarabine and cyclophosphamide or fludarabine and mitoxantrone have shown promise, but with substantial toxicity [128]. As in CLL, the combination of fludarabine and cyclophosphamide and rituximab (FCR) has yielded complete remissions. The role of high-dose therapy followed by hematopoietic stem cell transplantation is limited given the older patient population. However, younger patients can be treated with allogeneic transplantation with low-dose conditioning, especially when the patient has a HLA identical matched sibling [129].

A steady stream of new monoclonal antibodies or oral B-cell inhibitors have become available for treatment in the first line or relapsed setting which has shifted the treatment away from purine analog based regimens such as FCR. These new therapies will be discussed in detail in a separate chapter.

Three other indolent, low-grade NHL deserve further mention. Extranodal marginal zone lymphoma is another subtype of indolent, low-grade NHL. Localized gastric MALT lymphoma is unique because of its association with *H. pylori* and resolves with appropriate antibiotic treatment [130]. The presence of t(11;18) in the tumor cells predicts a poor response to antibiotic therapy [131]. Patients with localized disease and persistent lymphoma despite antibiotics can be effectively treated with rituximab [132]. Durable complete remissions are typical with radiotherapy. Nodal marginal zone lymphoma is a rare disease, mainly of older women. Most patients present with lymphadenopathy, often in the neck [133]. There is little consensus about treatment and the site that is involved and the age of the patient dictate management. The principles of therapy that were described for follicular lymphomas are usually the best treatment approach(es). Splenic lymphoma with villous lymphocytes represents another special lymphoma that presents with splenomegaly and B cells with villous projections in the blood. Treatment of hepatitis C with interferon-α alone or in combination with the antiviral agent ribavirin is associated with regression of splenic lymphoma [134]. Splenectomy was once used for diagnosis and treatment, but rituximab is highly effective and should be considered before splenectomy.

Aggressive Non-Hodgkin's Lymphoma

DLBCL is the most common, aggressive NHL and usually presents in the sixth or seventh decade, but also affects individuals of all ages. The presentation is quite variable, ranging from localized to disseminated disease, with or without nodal involvement. Primary mediastinal B-cell lymphoma (PMBL) represents a clinicopathologic variant of DLBCL, usually presented in young females as a mediastinal mass. DLBCL often frequently arises as a transformation from indolent NHL.

Unlike indolent NHL, radiation treatment alone is associated with high recurrence rates, thereby necessitating systemic chemotherapy. Early stage disease (I/II) has been subjected to clinical trials with or without radiation therapy. Initially, combined therapy became the standard based on an early trial showing an OS advantage for limited course CHOP plus involved-field radiation over CHOP [135]. Long-term follow-up unfortunately showed late systemic relapses in the combined-modality arm [136]. The improved outcomes with R-CHOP are now limiting the necessity of radiotherapy in early stage DLBCL. Advancements in PMBL treatment have also been shown to obviate the need for radiation in most patients with a disease that was once thought to require radiation. A dose-adjusted regimen of doxorubicin, vincristine, and etoposide infused over 96 h with bolus intravenous cyclophosphamide, rituximab, and oral prednisone (DA-EPOCH-R) has been showing promise [137]. For example, if the initial disease was less than 10 cm and post treatment PET scan is negative, radiation therapy can be omitted. In a more recent prospective trial with DA-EPOCH-R, in which 65% of patients had disease greater than 10 cm, there was an overall survival rate of 97% without radiation [138]. This was confirmed in much longer follow-up of a retrospective cohort. Omitting radiation may be important to eliminate the long-term toxicities, especially in younger patients.

As most patients with DLBCL present with later stage disease, systemic chemotherapy is the mainstay of treatment. The CHOP regimen is one of the oldest regimens in cancer therapy and was developed in the mid-1970s when anthracycline doxorubicin was added to the cyclophosphamide, vincristine, prednisone backbone [139]. Since the response rate was only 44%, many anticipated improvements were added to the CHOP platform in an attempt to improve response rates. Ultimately, these many attempts at modifying the regimen proved unfruitful as proven by a four-arm study evaluating 899 patients randomized to receive CHOP, m-BACOD, ProMACE-CytaBOM, or MACOP-B demonstrated that the 3-year estimates of time to treatment failure for these arms were 41, 46, 46, and 41%, respectively [140]. There were no risk groups according to the International Prognostic Index, as developed by Shipp et al. [79] that benefited from one of the four arms of the treatment trial compared with other arms.

As in the indolent NHL, rituximab has made the greatest impact on the treatment of DLBCL. Table 45.8 illustrates supporting trials. A study by GELA which showed CR rates (76% vs. 63%) and EFS rates (47% vs. 29%) favoring R-CHOP over CHOP [141, 142]. A U.S. intergroup and European MInT group study confirmed these findings in a similar patient population and R-CHOP became the standard of care in DLBCL [143, 144]. Since the establishment of rituximab's benefit in DLBCL, researchers have moved to test the effect of rituximab in dose-dense CHOP. The RECOVER-60 trials compared CHOP-14 with and without rituximab, randomized between six and eight cycles. No significant difference in outcomes was noted [145]. Although an excellent 3-year EFS rate of 66% was noted, it is difficult to extrapolate these results to the rituximab era. A definitive trial comparing R-CHOP 14 to R-CHOP 21 was completed showing no molecular or clinical subgroup benefited from the dose dense regimen [146]. R-CHOP remains the standard of care for the vast majority of DLBCL.

Table 45.8 Clinical trials for diffuse large B-cell lymphoma

Study	Therapy	Patient group	Event-free survival	Overall survival
Phase III	R-CHOP	Age ≥ 60 years	47% at 5 years	58% at 5 years
R-CHOP versus CHOP	GELA	All IPI		
Phase III	R-CHOP	Age ≥ 60 years	53% at 3 years	44% at 10 years
R-CHOP versus CHOP	U.S. Intergroup	All IPI		
Phase III	R-CHOP-like [*] MlnT	Age ≤ 60 years	79% at 3 years	93% at 3 years
Phase III	R-CHOP-14 (X6)	Age ≥ 60 years	66% at 3 years	78% at 3 years
R-CHOP-like versus CHOP		Low IPI		
Phase III	ACVBP	Age ≥ 60 years	39% at 5 years	46% at 5 years
Phase II, NCI	DA-EPOCH-R	All age ≥ stage2	79% at 5 years	80% at 5 years
Phase II, CALGB	DA-EPOCH-R	All age ≥ stage2	81% at 5 years	84% at 5 years
Phase II, Spain PETHEMA	DA-EPOCH-R	All age	48% at 10 years	64% at 10 years

While it is clear that rituximab has significantly improved the overall outcome of DLBCL, several studies suggest that its benefit is limited by tumor pathobiology [147, 148]. Rituximab's benefit was primarily in bcl-2-positive DLBCL, while another study showed that benefit was limited to bcl-6-negative DLBCL. These biomarkers likely relate to the new molecular taxonomy of DLBCL defined by gene profiling and suggest that rituximab might primarily benefit tumors that are derived from a postgerminal center B cell [149], and patients with both the germinal center B and activated B genetic subtypes benefit from rituximab [150].

There have been three phase II studies to report favorable outcomes using DA-EPOCH-R for DLBCL in general suggesting that tumor cell of origin (GCB or ABC) respond equally well. About 40% of patients in the two U.S. studies had IPI 3 or higher [151–153]. There have been no phase III studies with head-to-head comparison to R-CHOP and the exact subset of patients that benefit the most has yet to be determined.

The role of high-dose chemotherapy and Au-HCT in the initial treatment of DLBCL remains controversial [154, 155], since the studies that have suggested a benefit were performed in the pre-rituximab era [156]. In the most recent study with two-thirds of the patients receiving R-CHOP instead of CHOP, there was no survival benefit demonstrated [155]. However, it is widely accepted that Au-HCT is useful in second or third relapse, in the setting of chemosensitivity to additional salvage chemotherapy. Nevertheless, Au-HCT is also associated with late toxicities, including leukemia and secondary myelodysplasia, which must be considered in the risk benefit of treatment [157]. The role of Al-HCT is often limited, given the older age group of DLBCL, but has been employed with only modest success in younger patients due to high treatment-related mortality.

Burkitt's lymphoma (BL) occurs in the first two decades of life, is highly aggressive, and is derived from a germinal center B cell. The high apoptotic rate accounts for its "starry sky" appearance. Immunophenotypically it is CD20 positive, CD10 positive, and TdT negative [158]. Three clinical variants are recognized: endemic BL, which is found primarily in equatorial Africa; sporadic BL, which presents worldwide but is the most common type in Western countries; and immunodeficiency-associated BL, which is associated with HIV infection. There are important clinical differences in these variants. Endemic BL typically presents with jaw and facial bone disease and is virtually always associated with EBV. Sporadic BL usually presents with ileocecal disease and is associated with EBV in 30–50% of cases. Immunodeficiency-associated BL usually occurs in HIV-positive populations and is associated with nodal disease and is variably associated with EBV. BL may be associated with CNS involvement, particularly when there is bulky or disseminated disease.

BL requires emergent chemotherapy for all disease stages. The high tumor proliferation rate led to the use of dose-intense regimens with a short cycle time to theoretically minimize tumor regrowth between cycles [159]. Multiple drugs, administered in alternating combinations, are employed, and the high rate of spread to the CNS has led to the standard use of CNS prophylaxis [160, 161]. Though most BLs occur in children, Magrath and colleagues demonstrated that adults and children have a similar disease outcome when treated with the same regimen [162]. Toxicity is an important clinical limitation of these regimens in adults, particularly in older patients, in whom severe morbidity and even mortality occur. However, no significant modifications of the previous mentioned regimens have made significant improvements. Relapsed BL is extremely chemoresistant and is always uniformly fatal. Table 45.9 lists several treatment regimens for Burkitt's lymphoma [163–167] (Table 45.9).

MCL is a rare B-cell lymphoma and has a median age of 60 years and male predominance [40]. Its pathobiology is due to dysregulation of the cell cycle, with almost all cases showing the t(11;14) translocation and overexpression of cyclin D1 (bcl-1). Cyclin D1, CD20, and CD5 are virtually diagnostic of MCL. The aggressiveness of this disease can be anywhere from an indolent course to an aggressive and treatment refractory course. Using prognostic tools such as the MIPI and noting the proliferative index as measured by the ki-67 stain may help to tailor the most appropriate approach. One study showed a clinically meaningful ki-67 cutoff of 30% [168].

Table 45.9 Chemotherapy regimens for Burkitt's lymphoma

Therapy	Histology (number)	EFS rate	OS rate
LMB 89	Burkitt's and L3 ALL (420)	92% at 5 years	92% at 5 years
BFM 90	Burkitt's and L3 ALL (322)	89% at 6 years	14 deaths
CODOX-M/IVAC	Burkitt's	85% (children) and 100% (adults) at 2 years	2 deaths
CODOX-M/IVAC	Burkitt's (52)	65% at 2 years	73% at 2 years
CODOX-M/IVAC	Burkitt's (25)	84% at 2 years	89% at 2 years
Liposomal O			
Hyper-CVAD	L3 ALL	61% at 3 years for disease-free survival	49% at 3 years
R-Hyper-CVAD	Burkitt's and L3 ALL (28)	88% at 3 years	89% at 3 years
DA-EPOCH-R	Burkitt's (19 DA-EPOCH-R, 11 SC-EPOCH-RR)	95–100% at 6 years	90–100% at 6 years
Short course EPOCH-double dose R			
CALGB 10002	Burkitt's (105)	74% at 4 years	78% at 4 years

For patients presenting with advanced stage disease and median survival can range from 3–5 years. While OS is improving with the development of more effective therapies involving Au-HCT, cure remains elusive for most patients [83]. Treatment approaches are limited by the older median age of patients with MCL, but more aggressive regimens are used in younger patients. Fractionated cyclophosphamide administered with doxorubicin, vincristine, and dexamethasone (hyper-CVAD) alternating with high-dose methotrexate and cytarabine has shown great promise over traditional CHOP-based regimens [162, 169]. This effect was further consolidated with Au-HCT. As with other NHL, the CD20-positive nature of MCL made the addition of rituximab attractive. Hyper-CVAD with rituximab alternating with high-dose methotrexate and cytarabine was reported in untreated MCL [170]. Though the regimen was effective, the unacceptable toxicity in older patients led the authors to recommend it only in patients under 66 years of age. As such a modified dosing schedule for older patients is often employed, but has not been validated in a controlled clinical trial.

Several phase II studies support the role of consolidation with Au-HCT in first-line MCL patients after induction therapy [171, 172]. Incorporating Rituximab into the induction chemotherapy followed by autologous transplant for patients up to 69 years of age resulted in 64% 5 year overall survival [173]. Allogeneic stem cell transplantation with reduced-intensity conditioning has shown to be feasible in MCL [174].

For indolent forms of mantle cell lymphoma, several clinical trials have assessed the efficacy and safety of bendamustine and rituximab (BR) in patients with relapsed MCL [175]. Rummel and colleagues showed an RR of 90% and a 38% CR, PFS was 24 months. As previously discussed, the phase 3 study comparing BR to R-CHOP for first-line treatment of indolent mantle cell showed a ORR were similar (93.8% vs. 93.5%), but patients treated with BR had a significantly longer median PFS. Additionally, the BRIGHT study's mantle cell subgroup showed superiority in response rates with BR compared to R-CHOP [116].

The proteasome inhibitor bortezomib has shown promising activity in patients with relapsed or refractory MCL. After demonstrating activity in two small phase II studies, a large multicenter study reported a response rate of 33% with 8% CR in relapsed or refractory MCL [176, 177]. Combinations of bortezomib or rituximab and chemotherapy have been investigated in MCL. Another option in relapsed MCL patients is rituximab plus bortezomib. Preliminary results from a Phase II Study of VELCADE (bortezomib) in Combination With Bendamustine and Rituximab in Subject With Relapsed or Refractory Follicular Lymphoma (VERTICAL) showed excellent response rates with acceptable toxicities [178]. A phase III study of 487 patients with newly diagnosed mantel cell lymphoma not eligible for stem-cell transplant were given either R-CHOP or bortezomib substituted for vincristine (VR-CAP). At 40 months of follow-up there was a PFS advantage of 14 versus 25 months and a 4 year overall survival of 54 versus 64% [179]. Currently bortezomib is FDA approved for use in MCL.

Conventional Salvage Therapy

Once a patient has relapsed after initial chemotherapy for NHL, the ability to induce a remission with subsequent salvage therapy is reduced and the ability to cure the patient with conventional chemotherapy is less than 5% in most series. The salvage treatment should be approached in an individual manner. Although most relapsed aggressive lymphomas require combination chemotherapy, it is important to note that patients with local disease can be salvaged with radiation therapy. Initial historical regimens included IMVP-16 and MIME [180, 181]. These regimens have since evolved into MINE, ESHAP, and MINE-ESHAP [182]. Now there are a variety of active chemotherapy regimens for relapsed or refractory NHL [182–186] (Table 45.10).

Platinum-containing regimens, such as ESHAP and ICE, are currently among the most widely used types of salvage treatment. Salvage treatment should include different agents from past treatment to avoid drug resistance. The addition of rituximab appears to enhance the activity of salvage regimens as demonstrated by results with R-ICE and ICE, which showed a CR rate of 53% and 27%, respectively.

Patients with chemotherapy-sensitive disease have the best outcome with Au-HCT, and it is recommended at initial relapse; it has yielded OS and EFS rates in the range of 40–50% and 30–40%, respectively [185, 187]. Au-HCT rarely achieves cure in BL and lymphoblastic lymphoma, but is often successful in ALK-positive anaplastic large cell lymphoma (ALCL). NHL that are rarely cured with initial treatment, such as PTCL, are less likely to benefit from ASCT. It is important

Table 45.10 Salvage chemotherapy regimens

Therapy	Patient group	CR rate (%)	OR rate (%)	OS rate
DHAP	Relapsed or refractory lymphoma	31	58	25% at 2 years
ESHAP	Relapsed or refractory lymphoma	37	70	31% at 3 years
EPOCH	Relapsed or refractory DLBCL	36	70	30% at 6 years
R-ICE	Relapsed or refractory DLBCL	53	88	–
RGemOX	Relapsed or refractory DLBCL	34	43	41% at 1 year

CR complete response, *DHAP* dexamethasone, cytarabine, and cisplatin, *ESHAP* etoposide, methyl-prednisone, cytarabine, and cisplatin, *EPOCH* etoposide, prednisone, vincristine, cyclophosphamide, and doxorubicin, *OR* overall response, *OS* overall survival, *R-ICE* rituximab, ifosfamide, carboplatin, and etoposide

to consider that the incorporation in up-front therapy with rituximab has biased the relapsed population toward greater resistance. The incorporation of rituximab or radioimmunoconjugate into conditioning regimens can also enhance the benefit of ASCT, thus pointing out the importance of controlled ASCT trials in the post-rituximab era [188]. Of course, patients with chemotherapy-resistant disease do poorly with Au-HCT and should be considered for experimental treatments such as Al-HCT or clinical trials with novel agents or novel combinations. Agents such as lenalidomide have shown some promise in relapsed and refractory subtypes of NHL such as DLBCL and MCL [189, 190].

Uncommon NHL Classifications

Primary central nervous system lymphoma (PCNSL) is a rare and highly aggressive lymphoma that is confined to the CNS and is usually of diffuse large B-cell histology. Challenges in evaluation have been addressed in a report of an international workshop to standardize the criteria for baseline evaluation and response [191]. The incidence of PCNSL is particularly high in the setting of HIV infection, and is virtually always associated with EBV. In HIV, PCNSL is usually multifocal, while in HIV-negative patients it often presents as a solitary intracranial mass and is rarely associated with EBV. Treatment of PCNSL differs from that of systemic DLBCL because many chemotherapy agents do not adequately penetrate the blood–brain barrier. Combined modality with high-dose methotrexate and radiotherapy has been a mainstay of treatment because of better responses to either single modality [192, 193]. Unfortunately, such combined-modality treatment is associated with severe long-term neurotoxicity and there has been much interest in developing regimens that obviate or defer the need for radiation until relapse. The Bonn group and others have adopted such an approach and have reported promising results with chemotherapy and deferred radiation in younger patients [194, 195]. Studies are also addressing the role of immunochemotherapy with rituximab and other novel agents that promise to further improve the outcome of PCNSL [196]. Rituximab is of special interest, since it has been shown to cross the blood–brain barrier, especially with higher doses.

PTLD encompass a broad spectrum of diseases that occur in the setting of allogeneic hematologic and solid organ transplantation and show considerable heterogeneity [197]. It is approached by withdrawal of immunosuppression and administration of rituximab. Chemotherapy may be required if a clone develops which is immune independent [198]. Other similar clinical syndromes include methotrexate-associated lymphoproliferative disease (LPD) and EBV-associated LPD. Both of these entities are treated by withdrawal of the offending agent, if necessary, chemotherapy [199].

In the setting of HIV infection, many subtypes of NHL develop. Classifiably the incidence of PCNSL is increased 3600-fold and of BL is 1000-fold [200]. These can be AIDS-defining illnesses; however, a few other lymphomas can develop. These include Hodgkin's lymphoma, extranodal marginal zone lymphoma, and certain T-cell lymphomas [201, 202]. The introduction of HAART has had a dramatic and positive impact on the outcome of HIV-related lymphomas. Traditionally, treatment was less intensive in order to avoid further immunosuppression. Also HAART therapy was held to further reduce potential interactions and side effects. Through studies by the AIDS Malignancy Consortium and private investigators, it has been clearly shown that HAART therapy is quite beneficial during induction therapy for HIV-related lymphomas [203]. With better supportive care, treatment regimens have intensified to include R-CHOP without interruption of HAART therapy, with an appreciable improvement in RR. Currently, the same criteria for considering Au-SCT in patients with non-HIV lymphoma should be used as there is no difference in survival or non-relapse mortality [204].

There are a variety of T-cell or combined T-cell/NK cell disorders, approximately 14 subtypes, but are not well studied. They are relatively uncommon and account for approximately 15% of all NHL [205]. Generally, T-cell/NK cell lymphomas are clinically aggressive and incurable, with the exception of ALCL and early stage T/NK cell, nasal-type lymphoma [206]. ALCL is a CD30-positive T-cell lymphoma with an excellent prognosis and is highly curative, usually associated with the t(2;5)(p23;q35) translocation. This results in expression of the nucleophosmin anaplastic lymphoma kinase (ALK). ALK-positive ALCL tends to have a significantly better prognosis than do ALK-negative cases. In adults, CHOP-based therapy considered the standard of care, and all patients should be approached with curative intent. As with any T-cell lymphoma expressing CD30+, the drug antibody conjugate brentuximab vedotin can be used with success in the relapsed setting, potentially a 57% complete response in relapsed ALCL [207].

Extranodal T/NK cell nasal-type lymphoma is prevalent in Asia and the Central/South Americas. Universally, it is associated with EBV and has a predilection for the nasal cavity, nasopharynx, palate, skin, GI tract, and testis. Optimal chemotherapy is not well defined, but when localized, radiation therapy is quite effective [208].

Peripheral T-cell lymphoma is usually associated with a poor outcome. CHOP potentially with etoposide (CHOEP) is associated with good responses [209], but these are usually transient with almost universal relapse. Enrollment on clinical trials is highly encouraged as there are only a few moderately effective salvage agents each with around a 25–35% response rate such as alemtuzumab, bendamustine, romidepsin, belinostat, and pralatrexate [210–214]. Au-HCT after

induction with CHOP has shown good event-free survival, but is not curative. However, a graft-versus-lymphoma effect has been described and suggests a curative potential with Al-HCT.

Angioimmunoblastic T-cell lymphoma (AITL) is characterized by skin rash, organomegaly, hemolytic anemia, and systemic constitutional symptoms. Patients usually exhibit immunodeficiency in the form of dysfunctional polyclonal hypogammaglobulinemia. These features usually distinguish it from peripheral T-cell lymphoma. Optimal therapy has not been defined for AITL, but anthracycline-based regimens or cyclosporine has shown efficacy [215, 216]. Most patients succumb from progressive disease or infection.

Hepatosplenic T-cell lymphoma is another rare T-cell NHL which usually presents with marked hepatosplenomegaly and bone marrow involvement. Outcome is extremely poor and characterized by rapid progression and death. However, Al-HCT has been associated with prolonged remission and potential cure [217].

Conclusion

The last several decades have ushered an explosion of information which has directly translated into targeting care not only to individualized patients, but also to individualized subtypes of NHL. Just as rituximab marked a major therapeutic advance when added to CHOP chemotherapy, we are likely to see the impact of new B-cell targeted therapy. Unfortunately, T-cell NHL remains a challenging group of diseases with much room for improvement. With advances in treatment resulting in more long-term remissions, it is important to remember late complications such as anthracycline-related cardiotoxicity and secondary malignancies. The future of NHL treatment will involve better characterization to facilitate more targeted therapy.

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Non-Hodgkin Lymphoma of Childhood

46

Tony H. Truong, Sarah Alexander, and Sheila Weitzman

Introduction

Childhood non-Hodgkin lymphoma (NHL) is a diverse group of tumors derived from cells of lymphoid lineage. They occur as clonal proliferations at various stages of lymphocyte development. In contrast to adult NHL, childhood NHL is typically high grade although intermediate-grade (primary mediastinal B-cell lymphoma (PMBCL) and low-grade lymphoma can be seen. Current international cooperative group trials have resulted in cure of over 80% of children with NHL. Active research by these collaborative groups has advanced the understanding of the molecular mechanisms underlying the various diseases and has allowed for the addition of targeted therapies which are improving outcomes. Ongoing research into prognostic factors and further understanding of these molecular pathways will allow further improvement in survival and minimize the late effects in survivors of childhood NHL.

Epidemiology

Among childhood cancers, pediatric lymphomas account for 15% of cases and as a group are the third most common malignancy in children [1]. Approximately 50% of pediatric lymphomas are non-Hodgkin lymphoma, with the remainder being Hodgkin lymphoma [2]. Differences in incidence vary according to gender and race with NHL being commoner in males than females and approximately twice as common in whites than blacks among those less than 10 years of age [2]. The striking male to female preponderance (2.7:1) decreases with advancing age and varies among subtypes of NHL with incidence being approximately equal among male and female patients with primary mediastinal B-cell lymphoma [3, 4]. Worldwide, there is geographic variation in incidence with a strikingly high incidence in Cuba and Morocco (15 per million) compared to an incidence of 5 per million children in North America [5]. Endemic Burkitt lymphoma (BL) in central Africa accounts for almost half of all pediatric cancer in that region and the incidence reaches 50 per million children [5].

Etiology

Multiple genetic mutations within a single cell are required for malignant transformation. Like most neoplasms, lymphomas arise because of the inappropriate expression or loss of function of genes that regulate cell proliferation, differentiation, and programmed cell death (apoptosis). The malignant clone may result from increased proliferation of cells due to inappropriate expression of an oncogene such as *c-myc*, believed to be an important mechanism in high-grade pediatric lymphomas, or to alteration in genes, such as *bcl-2*, controlling apoptosis leading to decreased cell death, resulting in a more indolent clinical course, as is seen in adult low-grade lymphomas. The loss of a tumor suppressor gene expression and/or function, a mechanism common in many solid tumors, may also be seen in some patients with T-cell leukemia or lymphoma.

Viral infections may induce genetic changes and play a critical role in the pathogenesis of NHL. Examples of this include the role of EBV in the development of the endemic (African) Burkitt lymphoma (BL) and that of human T-cell leukemia/lymphoma virus (HTLV) I and II in the development of adult T-cell lymphoma or leukemia. The oncogenic potential of EBV and HTLV is increased by infection early in childhood. The EBV genome is found in the DNA of tumor cells in 95% of cases in endemic BL but in only 15% of sporadic cases [7, 8].

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The distribution of endemic BL coincides with the geographical malaria belt in Africa. Malaria stimulates B-cell proliferation and T-cell suppression, and the interaction of this chronic effect with EBV is believed to be a key factor in causing the translocation of the c-myc oncogene next to one of the immunoglobulin genes and thus in the pathogenesis of endemic BL [9]. The variation in incidence within this region, combined with case clusters and shifting foci of endemic BL, suggests the presence of additional cofactors such as concurrent infection with mosquito borne arboviruses and tumor promoters from local plant extracts [10].

The role of radiation in the development of lymphomas is not clear. Although there is an increased risk of NHL in patients treated with combined radiation and chemotherapy, there was no correlation of lymphoma incidence with increased radiation dose in atomic bomb survivors [11]. A possible association of pesticide exposure and NHL remains unproven. A French study of household exposure to pesticides suggested that insecticide use during pregnancy and paternal household use of pesticide were associated with an increased risk of childhood acute leukemia and NHL [12].

Abnormalities of the immune system, especially in T-cell-mediated immunity, pose an increased risk for the development of lymphoma. As many as 10% of children with congenital or acquired immunodeficiency develop NHL (Table 46.1), the highest incidence being in patients with ataxia-telangiectasia and Wiskott–Aldrich syndrome. In general, these patients tend to develop large-cell or Burkitt-type NHL, but not lymphoblastic lymphoma [13]. NHL is the commonest malignancy associated with the acquired immune deficiency syndrome (AIDS) and often occurs before the age of 4 years in those who are infected from a human immunodeficiency virus (HIV)-positive mother [14]. In AIDS patients, primary central nervous system (CNS) lymphoma is invariably associated with EBV [15]. Iatrogenic immunosuppression of patients undergoing allogeneic hematopoietic stem cell or solid-organ transplantation has resulted in a significant incidence of Epstein–Barr virus (EBV)-related and to a lesser extent of non-EBV-related posttransplant lymphoproliferative disease (PTLD). Uncontrolled EBV-induced B-cell proliferation secondary to T-cell dysfunction produces a spectrum of disease from polyclonal hyperplasia to true monoclonal malignant lymphomas.

Classification

Over the last 50 years, lymphoma classifications have evolved from being solely morphology-based to incorporation of molecular abnormalities inherent to such entities. The Revised European–American Lymphoma (REAL) [16] and recently updated World Health Organization (WHO) classifications [17] define the subtypes of lymphoma by a combination of morphology, immunophenotype, cytogenetic abnormalities, and clinical presentation. The histologic spectrum of pediatric NHL is considerably narrower than that of adult NHL, with the majority of childhood NHL being limited to four major categories (Table 46.2). By the WHO classification system, 40–45% of children with NHL have Burkitt lymphomas, 25% have T- or B-cell lymphoblastic lymphoma with T lineage predominating, 10% have anaplastic large-cell lymphoma (ALCL), and 10% diffuse large B-cell lymphoma (DLBCL), while the remainder are rare and unclassified subtypes [13]. Lymphoblastic lymphoma is morphologically and immunophenotypically indistinguishable from acute lymphoblastic leukemia (ALL) as reflected in the designation of T- or B-lymphoblastic leukemia/lymphoma in the WHO classification. An arbitrary cutoff of 25% marrow involvement distinguishes stage IV NHL from ALL, a distinction of questionable biologic significance. Nonetheless, this differentiation

Table 46.1 Incidence of malignancy in congenital immunodeficiency [6]

Syndrome	Tumor	Risk (%)	Percent of malignancies that are NHL
Ataxia-telangiectasia	NHL Leukemia, HL, adenocarcinoma	>12	41–66
Common variable ID	NHL, HL, leukemia, gastric carcinoma	8–10	45–62
Severe combined ID	NHL, HL, leukemia, occupational adenocarcinoma	5	31–76
X-linked agammaglobulinemia	Leukemia, NHL, HL, adenocarcinoma	6	33
Wiskott–Aldrich Syndrome	NHL, leukemia, HL	>10	59–75
Bloom syndrome	Leukemia, NHL, HL, adenocarcinoma	25	
X-linked lymphoproliferative disease (after EBV infection)	NHL, HLH, EBV and non-EBV-related LPD	24–35	
Selective IgA deficiency	NHL, gastric carcinoma, thymoma, other NHL, HL, adenocarcinoma	?	
X-linked hyperIgM syndrome			

ID immunodeficiency, *NHL* non-Hodgkin lymphoma, *HL* Hodgkin lymphoma, *EBV* Epstein–Barr Virus, *LPD* lymphoproliferative disease

Table 46.2 World Health Organization (WHO) classification of non-Hodgkin lymphoma commonly seen in childhood and adolescence [17]

Type	Frequency (%)	Subtypes
Burkitt lymphoma	40–45	• Burkitt lymphoma
		• Burkitt-like lymphoma with 11q aberration
		• High-grade B-cell lymphoma (HBCL)
Lymphoblastic lymphoma	20–25	• Precursor T-cell lymphoblastic lymphoma
		• Precursor B-cell lymphoblastic lymphoma
Diffuse large B-cell lymphoma	10	• Diffuse large B-cell lymphoma (DLBCL)
		• T-cell/histiocyte-rich large B-cell lymphoma
Anaplastic large-cell lymphoma	10–15	• Anaplastic large-cell lymphoma, ALK+
		• Anaplastic large-cell lymphoma, ALK−
		• Primary cutaneous anaplastic large-cell lymphoma
Other	<5	• Primary mediastinal (thymic) large B-cell lymphoma
		• Peripheral T-cell lymphoma, NOS
		• Posttransplant lymphoproliferative disorders (PTLD)

Data from Swerdlow SH, Campo E, Pileri SA, Harris NL, Stein H, Siebart R, Advani R, Ghielmini M, Salles GA, Zelenetz AD, and Jaffe ES. The 2016 revision of the World Health Organization classification of tumors of lymphoid neoplasms. Update of the current fourth edition. (Blood. 2016;127(20):2375–2390)

remains important for the accurate comparison of the results of therapeutic trials. All other types of lymphoma, such as the many subtypes of non-anaplastic peripheral T-cell lymphomas, comprise only 1–2% of pediatric NHL [18] but form the majority of the less common T-cell subtype of PTLD. Primary mediastinal B-cell lymphoma which, like the adult counterpart, is of intermediate grade, comprises under 3% of pediatric NHL [4].

Pathology

The majority of pediatric NHL are diffuse, aggressive, high-grade lymphomas, which tend to arise in extranodal lymphoid tissue such as thymus or Peyer's patches. The aggressive nature of most pediatric NHL is thought to reflect the growth rate of the counterpart normal lymphocyte precursor [18]. The stages of lymphocyte differentiation at which childhood lymphoma and leukemia are thought to arise [19, 20] are shown in Figs. 46.1 and 46.2.

This rapid growth rate is reflected by the presence of numerous mitotic figures. Macrophages surrounded by a clear area, giving a starry sky appearance, may be seen in all four types of childhood lymphoma, although it is most commonly associated with BL. DLBCLs contain large nuclei compared to their surrounding histiocytes. PMBCL is characterized by large lymphoid cells with abundant cytoplasm usually in the presence of sclerosis indicated by thick bands of collagen fibers. The morphology of LBL is indistinguishable from that of ALL, a homogenous proliferation of medium-sized cells with high nuclear/cytoplasmic ratio. Finally, a diffuse proliferation of large cells with abundant, faintly basophilic cytoplasm and pleomorphic, often reniform

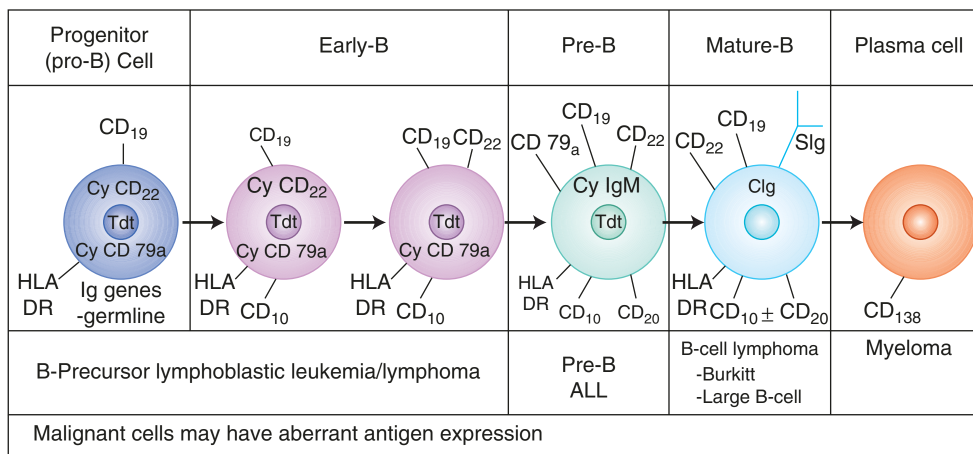

Fig. 46.1 Stages of B-lymphocyte differentiation. Malignant cells may have aberrant antigen expression. Data from [20] Rezuke WN, Abernathy EC, Tsongalis GJ. Molecular diagnosis of B- and T-cell lymphomas: fundamental principles and clinical applications. Clin Chem 1997;43:1814–23; Goust JM, Jackson A. Lymphocyte ontogeny and membrane markers. Immunol Ser 1993;58:161–86; and [21] Schmidt E, Burkhardt B. Lymphoblastic lymphoma in childhood and adolescence. Pediatr Hematol Oncol 2013 Sep;30(6):484–508)

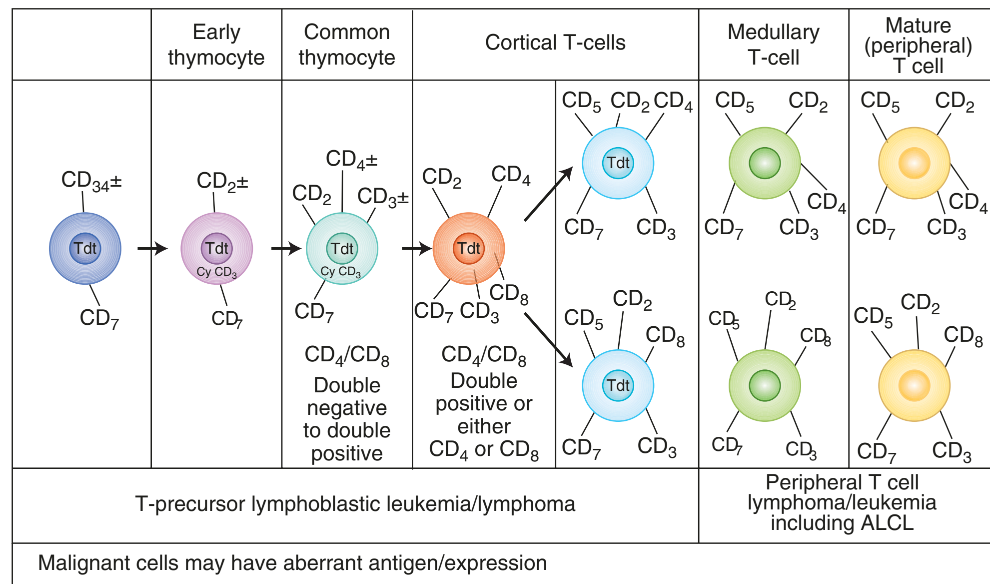

Fig. 46.2 Stages of T-lymphocyte differentiation. Malignant cells may have aberrant antigen expressions. Data from [20] Rezuke WN, Abernathy EC, Tsongalis GJ. Molecular diagnosis of B- and T-cell lymphomas: fundamental principles and clinical applications. Clin Chem 1997;43:1814–23; Goust JM, Jackson A. Lymphocyte ontogeny and membrane markers. Immunol Ser 1993;58:161–86; and [21] Schmidt E, Burkhardt B. Lymphoblastic lymphoma in childhood and adolescence. Pediatr Hematol Oncol 2013 Sep;30(6):484–508)

nuclei characterize ALCL. In addition to the four major types of NHL, children may present with a number of lymphoproliferative disorders, generally in the setting of congenital or acquired immunodeficiency.

Molecular Biology

The most common molecular alterations in NHL are rearrangements in immunoglobulin (Ig) and T-cell receptor (TCR) genes. Normally, rearrangement of Ig heavy and light chains and all four chains of the TCR occurs at precise times during differentiation. In B cells, heavy chain rearrangement occurs before light chain and κ light chain before λ. In T cells, γ- and δ-chain genes rearrange before α and β. DNA polymerase chain reaction (PCR) methods are used to identify these Ig and TCR gene rearrangements and thereby establish clonality.

Rearrangements of these receptor genes are not always lineage-specific. Ig gene rearrangements may be seen in T-cell disease, and TCR rearrangements in B-cell disease. Rearrangements involving light chain receptor genes tend to be more specific for B-lineage disease than heavy chain gene rearrangements, which may also be observed in other lineages [22]. PCR measurement of specific Ig rearrangements is being utilized to detect minimal residual disease (MRD) after induction chemotherapy in B-NHL [23], while PCR measurement of the protein product of the t(2;5) translocation in blood and bone marrow is being evaluated as a measure of minimal disseminated disease (MDD) at diagnosis and MRD after initiation of therapy in patients with ALCL [24]. In T-cell lymphoblastic lymphoma (T-LL), MDD and MRD can be evaluated by PCR amplification of genetic abnormalities and clonal TCR rearrangements or by flow cytometric methods [25] and these appear to be of prognostic significance [26].

Clinical Presentation

Pediatric NHL usually arises in lymphoid tissue, grows rapidly, and tends to spread, so that the majority of children present with locally advanced or metastatic disease. Only 10–15% of childhood NHL present with primary peripheral nodal disease and a minority present with involvement of extralymphatic tissue such as bone, skin, lung, and gonad. Involvement of the CNS presents as leptomeningeal (headache, neck stiffness, back pain) or cranial nerve palsies. Paraplegia due to spinal cord compression also occurs. There is a clear link between the different subtypes of NHL and their clinical presentation, described later in this chapter.

Diagnosis

The aggressive nature of most pediatric NHL stresses the urgency to obtain a diagnosis and begin therapy as soon as possible. Correct diagnosis requires morphology, cytochemistry, immunophenotyping, cytogenetics, and molecular genetics. Whenever possible, diagnostic tissue should be obtained from the most accessible site and large samples are generally preferred. On occasion, when the clinical situation limits the ability to obtain tissue under a general anesthetic, as in the case of a mediastinal mass, alternative tissue specimens may yield enough material for diagnosis and for limited additional studies such as cytogenetics or molecular genetics. This may include aspiration of pleural or pericardial fluid, bone marrow aspirate and biopsy, tissue biopsy

under local anesthetic and as a last resort fine-needle aspiration (FNA) of the tumor. In view of new biology-based approaches to therapy, however, fresh-frozen tissue is becoming increasingly important and excisional biopsies are often required to obtain adequate samples.

Before the patient is sent for biopsy, investigations to assess the extent of disease and of tumor-induced complications need to be made in a timely fashion. In particular, patients with large abdominal tumors need to be evaluated for gastrointestinal obstruction or perforation, as well as for renal impairment due to kidney infiltration, obstruction, or hyperuricemia. Patients with an anterior mediastinal mass need urgent evaluation for airway or superior vena cava (SVC) compression, right ventricular outflow compression, pleural effusion, and cardiac tamponade. It is important that these patients not be put under general anesthesia or into a supine position for the investigations, but instead procedures should be done with patients prone or lying on their side, and no patient with airway obstruction should be sedated for a procedure without consultation with an experienced anesthesiologist.

When anesthesia is considered to be too hazardous and diagnosis cannot be obtained from a peripheral node, pleural/pericardial fluid, or bone marrow, the mediastinal mass may be reduced in size by administration of corticosteroid therapy until a biopsy can safely be performed. An open biopsy must be performed as soon as possible, generally no longer than 24–36 hours after starting steroids, so that sufficient viable tissue is available for pathologic diagnosis and for important biology studies. A single-institutional review of 23 children with mediastinal lymphoma who received pre-biopsy steroids showed that 5 of 23 (22%) had a delay in diagnosis, failure in definitive diagnosis, or inaccurate staging as a result [27]. Appropriate prophylactic measures, such as the use of hydration and agents directed at reducing the risk of urate crystal formation, should be started early since steroid therapy may result in tumor lysis syndrome (TLS).

Staging and Risk Stratification

A comprehensive assessment includes complete blood cell count and differential, blood chemistry for renal and liver function studies, uric acid, potassium, phosphate, and calcium (tumor lysis syndrome) and lactate dehydrogenase (LDH) as a measure of tumor burden. Studies of the primary tumor include chest X-ray, computed tomography (CT) of neck, chest, abdomen, and pelvis (and occasionally head), ultrasound, and echocardiogram. Magnetic resonance imaging (MRI) scans are done for CNS and paraspinal disease, although limitations exist especially within the pediatric population. Extension of disease is determined by CSF examination and bilateral bone marrow aspirate and biopsy. Tests for CMV, VZV, hepatitis viruses, and EBV are recommended prior to therapy. HIV status should be assessed if clinically indicated.

The most widely used staging system for pediatric NHL is the St. Jude's system (Table 46.3), which is applicable to all major subtypes and separates patients with localized disease (stages I and II) from those with advanced disease (stage III and IV) [28]. Recently, a new Revised International Pediatric NHL Staging System (IPNHLSS) and Response Criteria was developed to address limitations of the former staging system, by incorporating new histiologic subtypes, giving recognition to sites of extranodal involvement, and improved diagnostic and imaging methods [29, 30].

Patients with stage IV NHL have bone marrow involvement with less than 25% blasts and/or have CNS disease; more than 25% tumor cells in the bone marrow is, by the WHO classification scheme, called leukemia. CNS disease is defined as blasts in cerebrospinal fluid, leptomeningeal infiltration, isolated cranial nerve palsy, or intracranial tumor masses. Isolated extradural disease which does not confer the same prognosis should be defined as stage III lymphoma, as should multifocal bone disease. The role of

Table 46.3 International pediatric non-Hodgkin lymphoma staging system

Stage	Description
Stage I	Single tumor with exclusion of mediastinum and abdomen (N; EN; B or S: EN-B, EN-S)
Stage II	Single EN tumor with regional node involvement
	≥Two N areas on same side of diaphragm
	Primary GI tract tumor (usually in ileocecal area), involvement of associated mesenteric nodes, that is completely resectable (if malignant ascites or extension of tumor to adjacent organs, it should be regarded as stage III)
Stage III	≥Two EN tumors (including EN-B or EN-S) above and/or below diaphragm
	≥Two N areas above and below diaphragm
	Any intrathoracic tumor (mediastinal, hilar, pulmonary, pleural, or thymic)
	Intra-abdominal and retroperitoneal disease, including liver, spleen, kidney, and/or ovary localizations, regardless of degree of resection (except primary GI tract tumor [usually in ileocecal region] involvement of associated mesenteric nodes that is completely resectable)
	Any paraspinal or epidural tumor, regardless of whether other sites are involved
	Single B lesion with concomitant involvement of EN and/or nonregional N sites
Stage IV	Any of the above findings with initial involvement of CNS (stage IV CNS), BM (stage IV BM), or both (stage IV combined) based on conventional methods

B bone, *BM* bone marrow, *EN* extranodal, *N* nodal, *S* skin

Data from Rosolen A, Perkins SL, Pinkerton CR, Guillerman RP, Sandlund JT, Patte C, et al. Revised International Pediatric Non-Hodgkin Lymphoma Staging System. J Clin Oncol 2015 Jun 20;33(18):2112–2118 [29]

fluorodeoxyglucose (FDG) positron emission tomography (FDG-PET) in staging and response evaluation is emerging as a powerful tool with increased sensitivity compared to CT, but its use for therapeutic stratification and modification is still being investigated [30, 32]. Significant differences in intensity and length of therapy as well as in prognosis for patients with localized and advanced-stage NHL emphasize the importance of accurate disease staging.

For risk stratification of mature B-NHL, a more useful clinically based grouping system was developed by the Lymphome Malins de Burkitt (LMB) group [33]. Group A (low-risk) defined as resected stage I and abdominal primary tumor completely resected stage II; Group C (high-risk) defined as bone marrow disease (≥25% L3 blasts) and/or CNS disease; Group B (intermediate-risk) comprises all others. In addition, an elevated LDH level at diagnosis that is above twice the upper limit of normal [33] or ≥500 U/L [34] continues to have negative prognostic significance among intermediate-risk patients and is currently used to stratify LMB Group B patients into high- and low-risk strata in an ongoing international B-NHL clinical trial. A similar "grouping" system is utilized by the Berlin-Frankfurt Muenster (BFM) study group which incorporates LDH levels within the R1-R4 grading system [35]. International Response Criteria have recently been developed to improve the assessment of treatment response based on contemporary imaging, flow cytometric and molecular techniques [30].

Treatment Approaches and Prognosis

Surgery is occasionally needed to deal with acute complications such as intussusception, bleeding, or bowel perforation but most importantly to obtain adequate tissue for diagnosis. Localized abdominal tumors seen at the time of laparotomy are often easily resected, and the prognosis is generally excellent. Surgery should not be performed for the purpose of resection or for debulking large tumors; in addition, surgical interventions that delay the onset of chemotherapy should be avoided. The place of surgery in the assessment of residual tumors postchemotherapy is controversial. The French Society for Pediatric Oncology (SFOP) demonstrated that 75% of residual abdominal masses were necrotic [33] and suggested that second-look surgery was necessary to define remission status. The success of intensification of therapy in patients with viable disease found by biopsy suggests that an accurate assessment of residual tumor may be useful and prognostic.

Radiation therapy (RT) has been relegated to a secondary role in the treatment of NHL. In patients treated with adequate chemotherapy, RT has been shown to increase both short-term and long-term morbidity without providing therapeutic advantage. RT is therefore omitted in most modern protocols except for patients with CNS disease. Even for patients presenting with a paraspinal mass and paraplegia, no advantage of addition of RT has been demonstrated, and RT should be limited to patients who fail to respond rapidly to chemotherapy [13]. Possibly because of rapid cell cycling, radiation given in single daily fractions is relatively ineffective in Burkitt lymphoma [36], although a later study suggests that hyperfractionation with at least three fractions per day may have some benefit [37]. This should be taken into account when total body irradiation (TBI) is considered for relapsed BL in the setting of hematopoietic stem cell transplantation.

Combination chemotherapy is the primary treatment modality for childhood NHL. The use of multiagent, intensive chemotherapy regimens, based on alkylators, anthracyclines, and high-dose methotrexate, has markedly improved survival for children with NHL. The treatment protocol should be based on the subtype and extent of disease. Supportive care measures to reduce the risk of TLS should be considered and instituted early at presentation [38].

Burkitt Lymphomas

Pathology

Burkitt lymphoma (BL) represents the neoplastic transformation of a relatively mature B-cell precursor that expresses surface immunoglobulin [39]. BL exhibits a diffuse monomorphic proliferation of cells with a high nuclear/cytoplasmic ratio and deeply basophilic cytoplasm containing lipid vacuoles. Single-cell necrosis with ingestion of debris by macrophages, leading to the starry sky pattern, is frequently present. The nuclei are similar to or slightly smaller than histiocyte nuclei, and the lymphoma cells have been categorized as small noncleaved lymphocytes. The cells in BL are fairly monomorphic in size and shape, with clumped or irregularly distributed nuclear chromatin and two to five nucleoli. Previously a category of disease named Burkitt-like lymphoma was utilized. In the Burkitt-like subtype, there is more heterogeneity in cell size, and nucleoli tend to be fewer and more prominent. There has been much controversy concerning the significance of Burkitt vs. Burkitt-like histology. Recent work from the BFM group, using gene expression analysis, is beginning to shed light on this distinction affirming that the majority of Burkitt-like lymphomas are molecularly identical to BL and can be treated with the same regimen. Flow cytometry and immunohistochemistry demonstrate B-cell markers CD19 and CD20 and IgM class surface immunoglobulin with light chain κ or λ restriction. CD10 (common ALL antigen) is usually present in BL. Terminal deoxynucleotidyl transferase (TdT) is negative in Burkitt lymphoma cells, reflecting their mature phenotype.

Molecular Biology

BLs have characteristic cytogenetic changes, which in 80% of cases involve translocation of the *C-MYC* proto-oncogene on chromosome 8 (involved in cell proliferation and differentiation) to the Ig heavy chain gene locus on chromosome 14 (t[8;14] [q24;q32]). In the remaining 20% of cases, *C-MYC* is translocated to the κ light chain gene locus on chromosome 2 (t[2:8] [p11;q24]) or to the λ light chain locus on chromosome 22 (t[8;22] [q24;q11]). Although the t(8;14)(q24;q32) is common to both sporadic and endemic BL, the breakpoints within the two genes are different. Both chromosomal translocations result in juxtaposition of *C-MYC* next to the highly active *Ig* gene, leading to deregulation and uncontrolled expression of the C-MYC protein [39]. Gene expression profiling can be used to classify mature B-NHL into BL and DLBCL with increased precision. With this technology, Burkitt-like lymphoma was found to group with histologically classical BL based on similar gene expression signatures and together comprising 63% of pediatric B-NHL [40]. There remains debate on whether *C-MYC*-negative cases can be considered as true BL. A recently identified subgroup of lymphomas that resemble BL morphologically but lack *C-MYC* rearrangement and known to have 11q alteration is considered a new entity in the revised WHO classification [17, 41]. Cytogenetic abnormalities have not been used for risk stratification, but data suggest that rearranged *MYC* (8q24), del(13q), +7q, del(17p), and abnormalities in 13q and 22q are independent predictors of poor prognosis among B-NHL [42–45].

Clinical Presentation

Despite a similar histologic appearance, the sporadic and endemic BL differ in their clinical presentation. The peak age for presentation is 11 years in the sporadic type (North American and European) and 7 years in the endemic (African) type with a male predominance of 2–3:1 in both [46].

In the sporadic type, 60–90% of BL present with abdominal disease, while the remainder arise from B lymphocytes in the Waldeyer ring lymph nodes (tonsils, adenoids), peripheral nodes, or the mediastinum [33, 47, 48]. BL arises most commonly from relatively mature B cells in Peyer's patches within the gastrointestinal (GI) tract, most commonly at the ileocecal junction. Gonadal and kidney involvement is common. Bowel obstruction is a common presenting symptom. Eighty percent of patients present with an abdominal mass or with abdominal pain, abdominal distension, nausea, and vomiting. A localized BL may act as the lead point for an intussusception, and the tumor is not infrequently diagnosed during surgery for acute appendicitis. Involvement of tonsil or adenoid may lead to airway obstruction, commonly associated with nontender cervical adenopathy.

Jaw involvement occurs in only 15% of patients with sporadic BL and then usually as part of multiple bone metastases, whereas endemic (African) BL commonly presents with jaw tumors (70%) as well as involvement of the GI tract and kidneys. Peripheral lymph node involvement is unusual in patients with the endemic form of the disease. Bone marrow and CNS involvement may be seen with BL. Endemic BL patients more commonly present with CNS disease (leptomeningeal, cranial nerve palsies, and paraplegia), whereas bone marrow disease occurs more frequently in patients with sporadic BL.

BL is an extremely fast growing malignancy with a cell cycle doubling time of 2–3 days and a high spontaneous rate of apoptosis [46]. Patients with these tumors have the highest risk of any malignancy of developing TLS before or after the start of therapy. The survival of patients with BL was very poor until chemotherapy protocols were designed to take advantage of the very rapid cell cycling.

Treatment

Treatment of Localized Burkitt Lymphoma

Survival of patients with localized BL and B-large-cell lymphomas exceeds 95% at 5 years of follow-up, permitting investigators to focus on reducing treatment-related morbidity [13, 49].

In the Pediatric Oncology Group (POG) 9219 trial, patients with stage I and II nonlymphoblastic disease treated with 9 weeks of cyclophosphamide–doxorubicin-vincristine–prednisone (CHOP)-based chemotherapy achieved an event-free survival (EFS) close to 90% and demonstrated that radiation therapy can be safely omitted even for bone disease [49]. A similarly excellent outcome was achieved by the international FAB/LMB 96 study with a 4-year EFS of 98% and overall survival (OS) of 99%, after two courses of COPAD chemotherapy, a regimen that does not include intrathecal therapy [50]. It is difficult to compare efficacy of the two protocols as the POG trial enrolled patients with stage I and II resected and unresected disease, whereas the FAB/LMB96 study enrolled only patients with resected disease (stage I or stage II); unresected disease was assigned to Group B. It appears that either strategy is acceptable for localized B-NHL. Regardless of the exact therapy, the patients who relapsed did so within a year from diagnosis.

Treatment of Advanced (Stage III and IV) Burkitt Lymphoma

During the last few decades major improvements have been seen in the cure rate of BL and mature B-ALL, from less than 30% in the early 1980s to close to 90% currently. Starting

with the St. Jude Total B therapy [28], chemotherapy protocols have been developed based on the very rapid growth rate of these tumors; these include short-duration, dose-intensified, rapidly cycling courses involving high-dose S-phase drugs that cross the blood–brain barrier. Most successful protocols combine high doses of cyclophosphamide given in divided doses over a period of 3–5 days in combination with high-dose methotrexate (MTX) as well as high-dose cytosine arabinoside (Ara-C), together with vincristine and with or without anthracycline. All protocols include a corticosteroid in the form of prednisone or dexamethasone.

Reports of the best outcomes include those of the Société Française d'Odontologie Pédiatrique (SFOP) LMB 89 protocol (Table 46.4). A total of 561 patients were allocated to three risk groups (A, B, and C) and therapy was adapted to each risk group. This protocol resulted in an OS of 91% at 5 years, 87% for stage IV, 88% for B-ALL, and an improvement in DFS for CNS-positive patients to 79% from 19% in the earlier LMB 81 study [33]. The 5-year DFS was 95% for patients with a low LDH compared to 87% for those whose LDH was increased twofold or more ($p < 0.001$), except in Group C patients in whom neither LDH nor bone marrow involvement was predictive of outcome. To build upon these excellent results, the SFOP, the Children's Cancer Group (CCG), and the United Kingdom Children's Cancer Study Group (UKCCSG) combined to conduct the French–American–British FAB/LMB 96 trial, which aimed at reducing therapy and minimizing toxicity while maintaining efficacy of LMB89. Among children with intermediate-risk B-cell NHL (Group B), a reduction to half-dose cyclophosphamide (1.5 g/m^2) in Induction Block 2 (COPADM1) and the omission of Maintenance Block M1 yielded a 4-year EFS of 91%, no different from the full dose arm [48]. Delay in therapy of >21 days between courses 1 and 2, however, significantly adversely impacted survival, stressing the importance of treatment intensity [51].

For Group C patients, the probability of 4-year EFS and OS was 79% and 82%, respectively. FAB/LMB96 randomized patients who responded following COPADM2 to either a standard arm or a reduced intensity arm, leading to a 4-year EFS of 90% vs. 80%, respectively, and early closure of the reduced intensity arm [31]. This dimimution in efficacy was noted in both CNS-negative (94% vs. 86%) and CNS-positive (84% vs. 72%) patients. Importantly, CNS-positive patients showed similar outcomes to LMB89 (EFS 79%) after high-dose methotrexate (8 g/m^2) and additional intrathecal chemotherapy while omitting cranial irradiation [31]. An important finding of this study was the delineation of groups with poorer outcomes who require intensification or alternative therapy in future protocols, such as those who responded poorly to the COP prephase (incomplete responders 78% vs. nonresponders 30% 4-year EFS), patients with combined bone marrow and CNS disease (61% 4-year EFS), and those with primary mediastinal diffuse large B-cell lymphoma (72% 4-year EFS) [48].

Other groups have produced similar results. In the BFM-NHL 86/90 and 95 studies, the BFM confirmed the safe omission of cranial radiation even for CNS-positive disease and the importance of high-dose methotrexate and high-dose Ara-C in advanced disease. They also confirmed that toxicity could be reduced and efficacy maintained by shortening intravenous methotrexate to 4 h vs. 24 h in those with limited stage B-NHL (Groups R1 and R2: stage I, II, and III with LDH < 500 U/L—failure-free survival (FFS) 95%) but not in patients with advanced disease (Table 46.5) [35].

Both the BFM and LMB groups showed that patients with residual disease following three courses of chemotherapy could be successfully treated with high-dose chemotherapy (HDCT) and autologous bone marrow transplant (ABMT). In BFM-90, only 1 of 6 patients suffered from disease progression and in LMB-89, 9 patients out of 12 achieved a CR

Table 46.4 Outcome for patients with advanced-stage Burkitt lymphoma

Protocol	Number	Stage or risk group	Outcome (%)	References
LMB 89	420	III/IV/B-ALL	87–93% 5-year EFS	Patte et al. [33]
		CNS+	79% 5-year EFS	
FAB LMB 96 Group B	451	I/II unresected	98% 4-year EFS	Patte et al. [48]
		III/IV, CNS−	85–90% 4-year EFS	
FAB LMB 96 Group C	204	IV, BM+, CNS−	88% 4-year EFS	Cairo et al. [31]
		IV, BM−, CNS+	82% 4-year EFS	
		IV, BM+, CNS+	61% 4-year EFS	
BFM-95	283	R1/R2	94% 3-year EFS	Woessmann et al. [35]
		R3/R4	81–85% 3-year EFS	
		CNS+	70% 3-year EFS	
COG ANHL01P1	45 Group B	Group B, III/IV	95% 3-year EFS	Goldman et al. [52, 53]
	40 Group C	Group C, all patients	90% 3-year EFS	
		BM+, CNS−	88% 3-year EFS	
		BM+, CNS+	93% 3-year EFS	
		BM−, CNS+	100% 3-year EFS	

Table 46.5 BFM-95 protocol for treatment of B-NHL

Drug	Dose	Day				
		1	2	3	4	5
Prephase V						
Dexamethasone orally/IV	mg/m^2	5	5	10	10	10
Cyclophosphamide IV 1 h	$200 mg/m^2$	x	x			
Methotrexate[a] IT	12 mg	x				
Cytarabine[a] IT	30 mg	x				
Prednisolone[a] IT	10 mg	x				
Course A						
Dexamethasone orally/IV	$10 mg/m^2$[e]	x	x	x	x	x
Vincristine V	$1.5 mg/m^2$[e]	x				
Ifosfamide IV 1 h	$800 mg/m^2$	x	x	x	x	x
Cytarabine IV 1 h	$150 mg/m^2$				x–x[b]	x–x[b]
Etoposide IV 1 h	$100 mg/m^2$				x	x
Methotrexate IV[c]	$1 g/m^2$	x				
Methotrexate[a] IT	12 mg	x				
Cytarabine[a] IT	30 mg	x				
Prednisolone[a] IT	10 mg	x				
Course B						
Dexamethasone orally/IV	$10 mg/m^2$[e]	x	x	x	x	x
Vincristine IV	$1.5 mg/m^2$[f]	x				
Cyclophosphamide IV 1 h	$200 mg/m^2$[e]	x	x	x	x	x
Doxorubicin IV 1 h	$25 mg/m^2$				x	x
Methotrexate IV[c]	$1 g/m^2$	x				
Methotrexate[a] IT	12 mg	x				
Cytarabine[a] IT	30 mg	x				
Prednisolone[a] IT	10 mg	x				
Courses AA[d,e]						
Methotrexate IV[c]	$5 g/m^2$	x				
Methotrexate[a] IT	6 mg	x				x
Cytarabine[a] IT	15 mg	x				x
Prednisolone[a] IT	5 mg	x				x
Course CC[d]						
Dexamethasone orally/IV	$20 mg/m^2$[e]	x	x	x	x	x
Vindesine IV	$3 mg/m^2$[h]	x				
Cytarabine IV 3 h	$3 g/m^2$	x–x[b]	x–x[b]			
Etoposide IV 2 h	$100 mg/m^2$			x–x[b]	x–x[b]	x[b]
Methotremate[a] IT	12 mg					x
Cytarabine[a] IT	30 mg					x
Prednisolone[a] IT	10 mg					x

IV indicates intravenously, *h* hour; *IT* intrathecally

Reproduced with permission from Woessmann W, Seidemann K, Mann G, et al. The impact of the methotrexate administration schedule and dose in the treatment of children and adolescents with B-cell neoplasms: a report of the BFM Group Study NHL-BFM95. Blood 2005; 105:948–58

[a]Doses were adjusted for children younger than 3 years. In courses A, B, AA, and BB, intrathecal therapy was administered 24 h after beginning of MTX intravenous infusion

[b]Doses are 12 h apart

[c]Patients were randomized to receive MTX as continuous intravenous infusion either over 24 h or over 4 h. In the 24-h arm, 10% of the MTX dose was given within 0.5 h, 90% of dose intravenously over 23.5 h. Racemic folinic acid (leucovorin) intravenously 15 mg/m^2 at hours 42, 48, and 54 after beginning of MTX. In courses AA and BB, the dose of leucovorin at hour 42 was 30 mg/m^2 intravenously. Adjustment of leucovorin dose in case of impaired MTX excretion as previously described [4]

[d]For CNS-positive patients, chemotherapy was applied intraventricularly as described in "Chemotherapy"

[e]Courses AA and BB are the same as A and B, respectively, with the exception listed

[f]Subdivided into three doses

[g]Maximum dose was 2 mg; vincristine was not given in patients of branch R1

[h]Maximum dose was 5 mg

after HDCT and ABMT [33, 34]. By comparison, four of five partial responders in BFM-86 treated with chemotherapy alone died. These results suggest that surgery to confirm residual tumor is necessary, as high-dose therapy may be successful for tumors that respond slowly but remain chemotherapy-sensitive. The role of PET-scans to allow avoidance of second look surgery has not yet been established.

Although it is generally accepted that cranial radiation does not improve results, the outcome for CNS-positive patients remains inferior at 82% compared to 88% 4-year EFS for bone marrow-positive/CNS-negative patients, and ≥90% 4-year EFS for the BM-positive/CNS-negative patients randomized to standard treatment (Table 46.4) [31]. Further intensification of methotrexate is currently being tested for this subgroup.

The addition of the anti-CD20 monoclonal antibody rituximab to chemotherapy is standard for all adult B-NHL but its value in pediatric CD20-positive NHL had not been definitively tested until recently. The COG study ANHL01P1 was a non-randomized pilot study designed to evaluate the safety of rituximab when added to Group B and Group C LMB-type therapy (Table 46.4) [53]. Results show that rituximab was well tolerated on the LMB backbone and though not designed to test efficacy, the best outcomes within Group B and C therapy were achieved with 3 year EFS of 95% and 90%, respectively [52, 53]. Even Group C patients with BM and CNS involvement, the worse prognostic group, showed remarkable improvement in 3 year EFS of 93%, in this pilot study.

The first interim analysis of the ongoing randomized Intergroup trial Inter-B-NHL Ritux 2010, evaluating the efficacy of rituximab in addition to standard LMB chemotherapy for patients with higher risk disease (group B/high LDH and group C) confirmed improvement in the rituximab arm,

resulting in an early 1-year EFS of 94% vs. 82%, mandating the early cessation of randomization in the study [54]. The addition of anti-CD20 antibody to chemotherapy is, therefore, now being considered standard of therapy for mature B-NHL in those countries where it is available.

Summary of the Findings of the Cooperative Group Trials

1. Advanced-stage Burkitt and B-cell ALL can successfully be treated with a short duration (3–6 months) of very intensive, multiagent alkylator-based chemotherapy.
2. Successive cycles of chemotherapy need to be given with minimal delay to prevent progression of these rapidly growing tumors.
3. Improvement in cure rate in advanced-stage disease occurred with increasing intensity of chemotherapy, particularly higher doses of methotrexate.
4. The addition of high-dose antimetabolite therapy, such as high-dose MTX and high-dose Ara-C, and intrathecal therapy is successful in prophylaxis and treatment of CNS disease, although CNS-positive patients have an inferior prognosis compared to CNS-negative patients. Intensification of methotrexate dose intensity through prolongation of the duration of high-dose MTX infusion is being tested by the LMB group.
5. Involvement of the bone marrow alone at diagnosis is no longer indicative of a poor prognosis. However, involvement of both CNS and bone marrow still carries an inferior prognosis.
6. A high LDH level at diagnosis remains an adverse prognostic factor in patients on intermediate risk therapy but not in the most intensively treated patients.
7. Most patients with BL who relapse will do so within the first 8 months after diagnosis. Patients with BL who survive disease-free for 10 months are likely to be cured.
8. Patients with residual, histologically proven disease after three therapy courses have an increased risk of disease recurrence, which is not reduced by local surgery or radiation, but may be improved by intensification of therapy.
9. Anti-CD20 monoclonal antibody therapy is emerging as an effective agent that can be added to traditional chemotherapy without significant additional toxicities and with improvement in EFS.

Lymphoblastic Lymphoma

Pathology

Unlike ALL, the majority of childhood lymphoblastic lymphomas (LBL) are of T-cell origin (85–90%) rather than B-precursor (10–15%) [55]. T-LBL and B-LBL appear histologically similar, but T-cell LBL most commonly presents with advanced-stage disease, whereas precursor B-cell LBL more often presents with localized disease and may present at unusual sites such as skin or bone or with disease below the diaphragm [56, 57].

LBL consists of cells that are morphologically indistinguishable from ALL. There is diffuse proliferation of medium-sized cells with a high nuclear/cytoplasmic ratio and scant amounts of basophilic cytoplasm (usually less basophilic than Burkitt lymphoma cells). The nuclear chromatin is finely stippled with indistinct nucleoli, and some degree of nuclear convolution may be seen in up to 50% of cases [58]. Mitotic activity is prominent and the starry sky histological pattern is commonly seen. The cells stain strongly for acid phosphatase; TdT is usually positive. By convention, LBL that involves more than 25% lymphoblasts in the bone marrow is called lymphoblastic leukemia and is indistinguishable from T or B-lineage ALL (B-ALL/T-ALL).

T-cell LBL is derived from thymic T cells most of which express the pan-T antigen CD7 as well as CD3. Other antigens that may be expressed depending on the stage of differentiation are CD1a, CD2, CD4, CD5 and CD8, and CD45RO, as well as other markers of immature T cells such as CD4 and CD8 double positivity or double negativity. CD10 (CALLA) is positive in about 50% of T-LBL cases [58].

Despite the morphologic similarity between LBL and ALL, subtle differences in the immunophenotype suggest that T-cell ALL may be derived from early to intermediate thymocytes, whereas T-LBL is more frequently derived from cells in the intermediate to late thymocyte stage [59]. Recent techniques using gene expression profiling have further enhanced the ability to distinguish between these two entities (see next section in this chapter).

Precursor B-cell LBL expresses the phenotype of common childhood precursor B-cell ALL (CD10, CD19, CD22, HLA-DR, TdT).

Diagnosis of T-LBL may be made on cytological and immunologic evaluation of pleural and/or pericardial effusions; however, care must be taken in the interpretation. Any large anterior mediastinal mass may, as a consequence of compression, result in the presence of thoracic duct lymphocytes, which morphologically resemble T lymphoblasts within pleural and pericardial fluid. The presence of TdT within the cells may be necessary for confirmation of the diagnosis. The combination of flow cytometric analysis and cell block immunohistochemistry on samples obtained by FNA, pleural or pericardial aspiration may obviate the need for biopsy in patients at high anesthetic risk [60]. Flow cytometry on CSF samples has been shown to improve detection of occult leptomeningeal disease in ALL and LBL. Adult patients who were CSF cytology negative but

FCM positive and who received standard CNS prophylaxis had a higher CNS relapse rate and lower 2-year OS, suggesting that these patients require more intensive CNS-directed therapy [61].

Molecular Biology

The study of chromosomal abnormalities in T-LBL has lagged behind that of T-ALL due to lack of adequate biopsy samples, nonetheless, chromosomal aberrations reported in T-ALL have been shown to be found in a proportion of T-LBL cases. Clonal TCR- rearrangements are present in most T-LBL and a proportion will demonstrate specific translocations of additional loci [25]. Many different translocations may be seen in T- LBL, usually involving translocation of a proto-oncogene to one of the TCR genes, α/δ, on chromosome 14 or less commonly, β, on chromosome 7, resulting in aberrant expression of the oncogene. The commonest translocations are t(11;14)(p13;q11) in 7%, t(10;14)(q24;q11) in 5%, and t(1;14)(p32–p34;q11) in 3% of cases [62, 63]. All the translocations found in LBL were previously reported in T-ALL except for t(9;17) which to date has only been found in T-LBL [21]. Studies utilizing genomic and gene expression analysis have shown both similarities and differences between T-LBL and T-ALL. In a recent study, activating NOTCH-1 mutations, found in more than 50% of cases of T-ALL, were found in 60% of T-LBL, suggesting a common pathogenetic mechanism for the two diseases. The study also found differences in expression of genes involved in chemotaxis and angiogenesis and the authors concluded that unique molecular characteristics drive the malignant T-cell precursors to colonize predominantly bone marrow or nodes [64]. Similarly, activating NOTCH 1 mutations were found in 60% of T-LBL patients in a BFM study and as previously reported by others was associated with a favorable prognosis while loss of heterozygosity (LOH) on chromosome 6q14–q24, found to be present in 25 pf 217 patients (12%), was associated with an unfavorable prognosis (EFS 27% vs. 86%) [65]. By contrast, mutations in the tumor suppressor gene FBXW7, responsible for degradation of the active protein intracellular NOTCH1 (ICN1) and found in 10–30% of T-ALL and T-LBL did not affect the 5 year pEFS in multivariate analysis [21]. LOH6q status will be used to identify very-high-risk T-LBL patients in future BFM trials [66]. Strategies that target NOTCH1 upregulation as well as mTOR pathways are currently being investigated in T-ALL and T-LBL [67].

In B-LBL, clonal rearrangements of the IgH gene are usually present and recurrent translocations more commonly seen in precursor B-ALL such as t(9;22)(q34; q11.2), t(12;21)(p13;q22), t(5;14)(q31;q32), and t(1;19)(q23; p13.3) may be found [68].

Clinical Presentation

T-LBL affects males more than females (2.5:1), with a peak age in the early second decade. Fifty percent of patients present with a mediastinal mass, often with pleural and/or pericardial effusions, right ventricular outflow obstruction, and/or a SVC syndrome. Associated symptoms include dyspnea, dysphagia, chest pain, and swelling of the face, neck, and arms. LBL are high-grade lymphomas and TLS may occur, especially if kidney infiltration is present. Urgent diagnosis and therapy are absolutely mandatory. Lymphadenopathy is present in 50–80% of patients and is usually supradiaphragmatic (cervical, supraclavicular, and axillary). Isolated or prominent abdominal disease is rare, although liver, spleen, kidneys, and abdominal lymph nodes may be involved. Bone marrow involvement is found in around 20% of cases, CNS involvement in around 5% and gonadal involvement may also occur [21].

B-LBL tends to present with localized disease and most frequently involves lymph nodes, skin, subcutaneous tissue, and bone, but may occasionally present with intra-abdominal disease. Bone marrow infiltration was found in 30% of patients with B-LBL in the BFM trials and CNS involvement in 5% [21]. B-LBL may occur as isolated skin disease often affecting the craniofacial area. Isolated skin B-LBL shares similar pathologic features to skin B-LBL that is associated with systemic disease and clinical staging is required for differentiation. By contrast, cutaneous T-LBL is usually associated with disseminated disease [69].

Treatment

Treatment of Localized Lymphoblastic Lymphoma

Around 15% of T-LBL presents with stage I or II disease. Early attempts to treat this subgroup with the short duration chemotherapy which was successful in localized mature B-NHL failed with a pEFS of 63% at 5 years in the POG 9219 trial, despite 6 months of continuation therapy with 6-mercaptopurine and low-dose methotrexate. However, an overall survival (OS) of more than 90% at 5 years was achieved by successful treatment for relapsed disease [49]. By contrast, the BFM group used more intensive therapy up-front and achieved an EFS of 92% in localized LBL, using the standard arm of the BFM T-cell ALL protocol [70, 71]. This protocol included standard BFM induction and a consolidation phase (protocol M) consisting of four doses of high-dose (5 g/m^2) methotrexate followed by maintenance therapy for 2 years. No reinduction therapy or local or cranial radiation was given for stage I and II patients.

Similarly, the COG A5971 study treated 60 patients with localized LBL, 75% of whom had B-LBL, with 24 months therapy based on CCG ALL-type therapy without cranial irradiation, and achieved a 5 year EFS and OS of 90% and 96%, respectively. No significant difference was found by stage (I vs. II), immunophenotype (T vs. B), elevated LDH, primary site or age. Unlike the POG 9219 study, patients who relapsed after this intensity of chemotherapy had a very poor salvage rate with four of five relapsed patients dying of disease. No CNS relapses occurred but patients with localized LBL continue to require CNS prophylaxis in the form of intrathecal chemotherapy.

The role of high-dose methotrexate (HDMTX) in localized LBL, utilized in the BFM and French LMT studies, but not in the equally successful EORTC studies, remains uncertain [72]. Based on the results of the COG A5971 randomized study (see discussion in next section), HDMTX is likely not needed in localized LBL.

Table 46.6 Outcome for patients with lymphoblastic lymphoma

Protocol	Number	Stage or risk group	Outcome (%)	References
Localized disease				
BFM-90/95	44	I/II	92% 5-year EFS	Reiter et al., Burkhardt et al. [56, 70, 71]
POG 9219	46	I/II	63% 5-year EFS	Link et al. [49]
COG A5971	60	I/II	90% 5-year EFS	Termuhlen et al. [72]
Advanced disease				
BFM-90	101	III/IV	90% 5-year EFS	Reiter et al. [70]
BFM-95	169	III/IV, CNS-	82% 5-year EFS	Burkhardt et al. [56]
COG A5971	254	III/IV	80–84% 5-year EFS (all arms)	Termuhlen et al. [77]
AIEOP LNH-97	114	III/IV	74% 7-year EFS	Pillon et al. [75]
SFOP LMT96	79	III/IV	85% 5-year EFS	Bergeron et al. [76]

Treatment of Advanced Lymphoblastic Lymphoma

Lymphoblastic lymphoma has been shown to respond optimally to protocols designed for T-ALL. Local radiation therapy, although effective, results in significant late risks, particularly when applied to the mediastinum, and is unnecessary if adequate chemotherapy is given [11]. All the current cooperative clinical trial group protocols have resulted in an EFS between 75% and 90% [73–77]. The results are summarized in Table 46.6.

Early studies demonstrated the importance of methotrexate intensification [73], and of the addition of l-asparaginase [74]. In 2000, the BFM group published the results of the BFM-90 protocol for patients with advanced-stage (III and IV) T-LBL using 24 months of therapy including a 6-week reintensification block. They achieved a 90% EFS at 5 years for 101 patients with T-cell LBL treated on this protocol without local radiotherapy [70]. Therapy was assigned based on stage, but patients who failed to respond adequately to induction therapy by day 33 as defined by less than 70% reduction in mass, more than 5% blasts in the BM, and/or persistent blasts in the CSF were reassigned to a high-risk ALL protocol (eight patients). The follow-up BFM-95 study showed that omission of prophylactic cranial radiotherapy for patients with CNS-negative stage III or IV T-LBL did not result in increased CNS relapses when four courses of high-dose methotrexate (5 g/m^2) were administered [56]. A contemporaneous COG study (A5971) evaluated CNS prophylaxis and early intensification of therapy with cyclophosphamide and anthracycline in patients with CNS-negative stage III or IV T-LBL on BFM-90-like chemotherapy; 254 patients were randomized to either four doses of 5 g/m^2 MTX with no IT-MTX in maintenance or intensified intrathecal methotrexate. The 5-year EFS for the four arms was 80%, 81%, 80%, and 84%. Age less than 10 years and radiologic response at 2 weeks was associated with improved EFS and OS [77].

Patients with CNS disease at diagnosis had 5-year EFS of 63% despite 4 doses of high-dose MTX and cranial irradiation, with the failures being due to relapse and not toxicity, however, as with earlier trials the small number of patients with LBL with CNS disease at diagnosis do not allow for further conclusions [77].

The cooperative group studies also showed that most of the relapses occur early and the optimal duration of therapy for advanced-stage LBL remains between 18 months (SFOP stages I-III) and 24 months (BFM, COG, AIEOP, SFOP). The EURO-LB 02 trial showed no difference in EFS for 119 patients randomized to 18 vs. 24 months [21]. The same study as well as the randomized EORTC CLG study demonstrated that attempts to improve relapse-free survival by replacing prednisone with dexamethasone in induction failed due to excessive treatment-related mortality in the dexamethasone arms [21, 78].

The role of high-dose MTX in advanced-stage CNS-negative LBL remains controversial. The COG A5971 randomized study showed no survival advantage to high-dose MTX as compared to intensified IT MTX but the latter patients received 11 doses of IT MTX during maintenance, compared to no IT therapy in maintenance for the high-dose MTX group [77]. A comparison of the short- and long-term toxicities of the two therapies would be of great interest but that data is not available.

The use of specific anti-T-cell therapy is currently under investigation. A recently closed COG phase III study tested the addition of nelarabine, a prodrug of Ara-G shown to be toxic to T lymphoblasts through the accumulation of Ara-G nucleotides, to a BFM backbone in high-risk patients with T-ALL but the results are not yet published. The current phase III COG study is evaluating the addition of bortezomib, a selective proteasome inhibitor, to an augmented BFM backbone for patients with T-ALL and T-LBL. If successful, these or similar anti-T-cell specific drugs may be added to the therapy of T-LBL.

Prognostic Factors in T-LBL

The poor salvage rate of patients with advanced-stage T-LBL has resulted in attempts to define subgroups of patients who may require intensified therapy.

Of the clinical parameters only age at diagnosis was found to be prognostic with patients less than 10 years of age having a superior outcome in the BFM, COG A5971 and St Jude Children's Research Hospital (SJCRH) trials. In the SJCRH study this difference was less apparent in the most recent era, suggesting that the more intensive therapy in the current protocols may eliminate the prognostic significance of age [79]. In the COG A5971 trial CNS disease at diagnosis resulted in a significantly poorer outcome but the numbers were too small for firm recommendation [77]. Patients with CNS disease continue to receive more intensive therapy with the addition of high-dose MTX, extra intrathecal therapy and cranial irradiation.

There are currently no reliable clinical prognostic factors for patients with advanced LBL. However, some promising biologic parameters which may be helpful in the future. The ability to evaluate minimal disseminated disease (MDD) by flow cytometry in T-LBL is emerging as a useful prognostic tool [25]. If these results are confirmed in further studies, MDD testing could identify a high-risk subgroup of patients for therapy intensification.

The BFM group have reported that loss of heterozygosity at chromosome 6q14-q24 is significantly associated with an unfavorable prognosis (EFS 27% vs. 86%, $P < 0.0001$) while NOTCH1 mutations were associated with a favorable prognosis (EFS, 84% vs. 66%; $P = 0.021$) [65]. Both prognostic markers will be used as stratification criteria in future BFM trials for lymphoblastic lymphoma.

Finally, early response to therapy is emerging as a prognostic factor.

The EORTC showed that clinical and radiological response to the 7 day prednisone prephase was significantly prognostic, but this needs to be confirmed by other study groups. The COG A5971 study showed that the radiologic response at 2 weeks significantly correlated with EFS and OS. The presence of a residual mediastinal mass at day 33 or at the end of induction was not found to be associated with a decreased survival in the BFM90–95 studies, however, all patients with <70% reduction at end induction had had therapy intensified [18]. It is as yet unclear whether a positive PET-FDG scan in a residual mass at end induction will improve the prognostic value of this finding by helping to differentiate active from necrotic tumor.

Early results suggest that minimal residual disease (MRD) testing by flow cytometry for aberrant immunophenotype and real-time quantitative PCR (RQ-PCR) for clonal TCR-gene rearrangements may be useful in response assessment [80]. To date, however, the place of MDD and MRD assessment in risk stratification remains investigational.

The presence of MDD at diagnosis of T-LBL, that is the detection by flow cytometry of lymphoblasts circulating in peripheral blood despite morphologically negative bone marrow has been shown by the COG and the Italian AIEOP group, to be significantly prognostic of relapse. Recent COG studies have incorporated MDD at diagnosis into risk stratification with the high risk group (>1% $CD3^+$, TDT^+ Cells in peripheral blood at diagnosis) being randomly allocated to the addition of nelarabine and now bortezomib to a standard ALL–type chemotherapy backbone.

B-Lymphoblastic Lymphoma

Because of small patient numbers, the correct treatment for B-lineage LBL (B-LBL) has not been defined in pediatrics. Patients with B-LBL treated with BFM-95 ALL-type therapy achieved a 5-year EFS of 92% [56]. Similar results were reported in the LMT and EORTC trials [81]. Patients with B-lineage LBL should be included on T-cell LBL or ALL-directed regimens and treated for 18–24 months.

Summary of the Findings of the Cooperative Group Trials

The findings of the Cooperative Group protocols may be summarized as follows:

1. Intensive ALL-type therapy results in an EFS of 90% in localized T and B-LBL.
2. Current intensive protocols have improved EFS to 80–85% for advanced-stage T LBL.
3. High-dose MTX (5 g/m^2 per dose) for at least four doses or intensified intrathecal therapy appears to be equally efficacious in advanced-stage T-LBL.
4. L-Asparaginase therapy was shown to be important in POG studies.
5. Local radiation therapy is not indicated if adequate chemotherapy is given.
6. Prophylactic cranial irradiation may be omitted in CNS-negative patients whose therapy includes steroids, intensified intrathecal chemotherapy, and/or high-dose MTX.

7. The SFOP and BFM studies suggest that even in patients with testicular disease at diagnosis, testicular radiation is only indicated for residual disease after high-dose MTX.
8. The length of the maintenance therapy as well as the optimal maintenance drugs remains unclear, but most current protocols utilize ALL-like therapy for a total duration of 18–24 months.
9. There are no clinical parameters including age, gender, site of disease, or LDH which are useful for risk stratification in LBL. Minimal disseminated disease and minimal residual disease testing appear to be promising tools for future studies. The presence of LOH 6q14-q24 suggests the need for therapy intensification while the presence of activating mutations of NOTCH1 is a marker for improved outcome.

Diffuse Large B-Cell Lymphoma

Pathology

Large-cell lymphomas (LCLs) are a heterogenous group of tumors in which the cells contain nuclei that are larger than those of the surrounding histiocytes. Major subtypes of LCL are diffuse large B-cell lymphoma (DLBCL), anaplastic large-cell lymphoma (ALCL), and the other, rarer, peripheral T-cell lymphomas, so-called because of their mature T-cell phenotype.

DLBCLs comprise about 10% of childhood NHL and are the most common subtype among patients with immunodeficiencies. The cells are of mature B-cell phenotype, express CD20, and show light chain restriction (either κ or λ). Surface expression of immunoglobulin may be absent in up to one-third, however [58]. In keeping with the mature B-cell phenotype, they are TdT-negative. Occasional cases of B-cell large-cell NHL with an extensive T-cell reaction (T-cell-rich B-cell lymphoma) have been described [58].

According to the WHO classification, primary mediastinal large B-cell lymphoma (PMBCL) is a rare distinct entity from DLBCL, and only accounts for 2% of all pediatric NHL [4, 17]. PMBCL with sclerosis is characterized by large lymphoid cells with abundant cytoplasm, a vesicular chromatin pattern, and prominent nucleoli. Sclerosis may vary from thick bands of collagen to fine spider-like sclerosis surrounding small groups of cells. The major differential diagnosis involves nodular sclerosing HL and ALCL.

Molecular Biology

Specific recurrent cytogenetic abnormalities are only seen in a minority of patients with DLBCL, although complex cytogenetic findings are common. Some pediatric large-cell lymphomas show the t(8;14)(q24;q32) translocation, which is identical to that seen in Burkitt lymphoma with a similar mechanism of transformation. Unlike in adults, translocations involving *BCL-2*, such as t(14;18)(q32;q21), are rarely seen in childhood DLBCL [82]. Gene expression profiling of adult DLBCL cases suggests the existence of three subgroups: germinal-center B-cell-like, activated B-cell-like, and intermediate group type 3, of which the best outcome is associated with germinal-center subtype. In the pediatric population, gene expression analysis has shown that DLBCL comprises mainly the germinal-center subtype (CD10+ or CD10−/BCL-6+/MUM-1−). These findings in conjunction with the absence of t(14;18) involving the *BCL-2* gene may explain the favorable outcome in children compared to adults [82]. A study which combined gene expression profiling, comparative genomic hybridization (CGH), FISH, and immunohistochemistry showed that one-third of histologic DLBCL were reclassified as BL and only 1 of 14 non-BL mature B-NHL cases showed *MYC* rearrangements. While no difference in survival was shown between these biologic differences, they may provide targets for future therapies [40].

PMBCL is characterized by expression of CD45, CD20, and CD79a, and a diffusely positive pattern for CD30, and recent gene expression studies show an overlap with Hodgkin lymphoma rather than DLBCL [83].

Clinical Presentation

Diffuse large B-cell lymphoma can present in any anatomic site and may present with abdominal disease similar to Burkitt lymphoma or a mediastinal mass similar to lymphoblastic lymphoma. The mediastinal location of primary mediastinal large B-cell lymphoma and the extensive sclerosis associated with it may result in a clinical presentation of airway compromise or SVC syndrome similar to that of lymphoblastic lymphoma. Bone marrow is less frequently involved than in the other types of NHL, and CNS disease is uncommon.

Treatment

The biologic heterogeneity of this subtype of NHL has made the determination of optimal therapy for this group of patients difficult. This is reflected in the fact that the POG studies have treated all patients with early and advanced-stage LCL on the same chemotherapeutic protocols, irrespective of subtype [84, 85]; in contrast, the COG and European study groups have combined all B-cell lymphomas and treated DLBCL and Burkitt lymphoma on the same short, intensive protocols and showed equivalent results for DLBCL and Burkitt lymphoma (Table 46.7) [31, 48].

For localized disease, three courses of CHOP chemotherapy as per POG 9219 achieved a 5-year EFS of 98% and OS of 98% among 46 patients with stage I and II DLBCL [85]. Similarly, the FAB/LMB 96 study achieved a 4-year EFS of 98% and OS of 99% in patients with localized NHL, 57 of whom had DLBCL, and none of whom had any events [50].

Table 46.7 Outcome for patients with diffuse large B-cell lymphoma

Protocol	Number	Stage or risk group	Outcome (%)	References
Localized disease				
POG 9219	46	I/II	98% 5-year EFS	Link et al. [85]
FAB LMB 96 Group A	57	Stage I/II completely resected	100% 4-year EFS	Gerrard et al. [50]
Advanced disease				
POG 9315	73	III/IV	64% 4-year EFS[a]	Laver et al. [84]
FAB LMB 96 Group B	145	DLBCL	93% 4-year EFS	Patte et al. [48]
	32	PMBCL	72% 4-year EFS	
FAB LMB 96 Group C	21	IV, BM+/CNS + among responders	90% 4-year EFS (reported no different than BL)	Cairo et al. [31]
BFM-95	81	Group R3	85% 3-year EFS	Woessmann et al. [35]
		Group R4	81% 3-year EFS	

[a]Outcome in treatment arm APO (doxorubicin, vincristine, prednisone) was not different from the experimental arm IDM/HiDAC (intermediate-dose methotrexate/high-dose cytarabine), EFS 64% vs. 70%, respectively

In intermediate-risk (Group B) DLBCL, the FAB/LMB 96 trial demonstrated a 4-year EFS of 93% [48] while the BFM group reported a 3-year EFS of 85% and 81% for all patients in risk groups R3 and R4, respectively, indicating no difference between patients with BL and DLBCL [35].

Patients with DLBCL appear to do well with either short, intensive alkylator-based therapy or longer, less intensive anthracycline/steroid-based therapy.

Primary mediastinal large B-cell lymphoma carries a poorer prognosis in pediatrics compared to adults, possibly because of the omission of involved field radiation therapy (IFRT) in children. Within pediatrics, patients with PMBCL treated on three successive BFM studies had a lower 5 year EFS of 70% when compared to DLBCL [4]. In patients with intermediate-risk disease treated on FAB/LMB96, those with PMBCL had a lower 5-year EFS of 66% compared with 85% among those with DLBCL [86]. Adult studies suggest that patients have an improved outcome on intensified chemotherapy protocols compared to CHOP [87, 88]. The NCI dose-adjusted etoposide, prednisone, vincristine, cyclophosphamide, doxorubicin, and rituximab (EPOCH-R) protocol has resulted in significantly improved 5 year 93% EFS and 97% OS in adult PMBCL without IFRT [89]. This same regimen is currently being tested in a phase II setting for pediatric PMBCL in the ongoing Inter-B-NHL Ritux 2010 Clinical Trial.

Summary of the Findings of the Cooperative Group Trials

1. Patients with large B-cell lymphomas do well on either a short intensive Burkitt lymphoma-like regimen or a longer, less intensive regimen. Most groups are including DLBCL patients on BL protocols, including the international COG-European B-NHL study (Inter-B-NHL Ritux 2010), which initial results have shown that the addition of rituximab is superior, leading to the early cessation of randomization for mature CD20 positiv e NHL.
2. In the BFM and SFOP studies, intermediate- or high-dose MTX with (BFM) or without (SFOP) intrathecal therapy effectively prevents CNS relapse without cranial irradiation. Local radiotherapy appears to be of little value in DLBCL, even when bulk disease is present.
3. As in Burkitt lymphoma, residual tumor after chemotherapy is usually necrotic, but should be confirmed following resection. If residual lymphoma is detected, intensified, systemic therapy rather than local radiation therapy is indicated.
4. PMBCL appears to have a better prognosis with intensive chemotherapy and the benefit of the addition of rituximab in adults has yet to be confirmed in pediatric studies.

Anaplastic Large-Cell Lymphoma

Pathology

Anaplastic large-cell lymphoma accounts for 10–15% of pediatric NHL. The histopathologic characteristics include cells with eccentric kidney shaped nuclei often with an eosinophilic area near the nucleus. There are currently five morphologic subcategories of ALK-positive ALCL included in the 2008 World Health Organization (WHO) classification system including common, small cell, lymphohistiocytic, Hodgkin-like, and composite [90]. ALK-negative ALCL, a much rarer diagnosis in children is considered a provisional entity in the current WHO system [17].

ALCL is uniformly CD30 positive. T-cell antigens are commonly expressed including CD2, CD4, and CD5. CD3 and CD8 are often negative. Some cases are described as having a “null cell” phenotype with absence of T cell markers however recent studies suggest a T-cell lineage based on gene signature [91]. The “null cell” phenotype is of no clinical or prognostic significance. Epithelial membrane antigen (EMA) is generally positive, and CD25 (IL2-R) is often expressed.

Molecular Biology

Unlike adult ALCL where only 40–50% are ALK positive, in children more than 90% have disease that harbors a

translocation involving ALK The most common translocation, t(2;5)(p23;q35) relocates a promoter sequence of the nucleolar phosphoprotein encoding gene (*NPM*) on 5q35 to the anaplastic lymphoma kinase gene *ALK* on 2p23, results in production of the fusion protein NPM-ALK and accounts for 90% of pediatric ALK-positive ALCL [92]. Other chromosomal rearrangements, such as inv 2 (p23;q35) and t(1;2)(q25;p23), as well as variant traslocations with partner genes on chromosomes 3, 9, 17, 19, and 22 also result in ALK expression through novel fusion partners [93]. The ALK protein can be recognized immunohistochemically on fixed tissue using an anti-ALK1 monoclonal antibody. The pattern of staining is concordant with the specific translocation, with only the NPM-ALK fusion showing both cytoplasmic and nuclear and nucleolar staining, while variant translocations, including t(1;2)(q25;p23) have ALK staining restricted to the cytoplasm [92]. Expression of ALK is normally confined to cells of the nervous system and is not detected in normal lymphocytes. Overexpression of *ALK* leading to inappropriate phosphorylation of intercellular growth regulators and inducing cell transformation has been shown to be essential for lymphoma growth and survival [94].

Clinical Presentation

Most children with ALCL present with advanced-stage disease involving nodal areas, including mediastinum, intra-abdominal and peripheral nodes and extranodal sites such as liver, bone, skin, and soft tissue [95]. Bone marrow involvement detected by morphology occurs in 10–15% of patients whereas detection by PCR for NPM-ALK occurs in approximately half of patients tested CNS involvement is rare occurring in approximately 3% of children. Very rarely ALCL can present in a leukemoid phase which can associated with diffuse pulmonary infiltrates.

Unlike the other lymphomas, lymphadenopathy due to ALCL may be tender to palpation and is more commonly associated with B symptoms, including fever, night sweats, and unexplained weight loss, as well as laboratory findings of, a high leukocyte count and thrombocytosis, all of which are thought to be due to excess cytokine production by lymphoma cells. ALCL must be included in the differential diagnosis of persistent fever and lymphadenopathy, particularly as a waxing and waning course may be seen, followed by rapid progression. This spontaneous regression may be mediated by cytokines such as TGF-β [96], and the rapid progression that may follow is thought to be due to escape from the cytokine-mediated effects.

Primary Cutaneous ALCL

Isolated cutaneous ALCL is usually ALK negative. Pathologically it can be indistinguishable from lymphomatoid papulosis. Primary cutaneous ALCL has a favorable prognosis and can be managed with initial observation only. Surgical excision or low-dose radiation have been used with excellent results for localized disease [97]. Low-dose oral methotrexate and more recently both vinblastine and Brentuximab vedotin have been shown to be of benefit in multifocal cutaneous disease [98, 99]. The presence of ALK protein indicates the necessity to treat as systemic ALCL [100].

Treatment

Treatment of Localized ALCL

The prognosis for the relatively rare patients with low stage disease, and in particular resectable disease, is very favorable. Six weeks of CHOP-based chemotherapy in POG protocol 9219 achieved a 5-year EFS of 84% and OS of 100% in patients with stage 1 and II ALCL [85]. The BFM group reported a 5-year EFS of 100% for nine patients with completely resected stage I and II disease with three courses of inpatient chemotherapy whereas the 5-year EFS was 73% for non-resected stage II and stage III disease with six courses of inpatient chemotherapy [101]. In a report on 36 patients with stage I disease on the ALCL99 study, those who had completely resected disease and received short course chemotherapy had an EFS of 100% whereas those with incompletely resected disease had a relapse rate of 30%, similar to patients with higher stage disease [102].

Treatment of Advanced ALCL

The majority of patients with ALCL present with stage II, III, or IV unresectable disease. Over the last two decades multiple clinical trials employing strategies of varying durations of therapy, specific combinations of drugs, dose intensity, and cumulative doses have demonstrated similar outcomes with EFS rates between 70 and 75% (see table below).

The Pediatric Oncology Group and subsequently the Children's Oncology Group conducted trials in children with ALCL utilizing the "APO" regimen (see Table 46.8 below). This regimen can be given in the outpatient setting with a cumulative anthracycline dose of 300 mg/m^2 an no alkylator therapy. It includes 6 doses of intrathecal methotrexate. The duration of therapy is 52 weeks. Modifications to the backbone with dose intensification of methotrexate

Table 46.8 Doxorubicin–prednisone–vincristine (APO) protocol for anaplastic large-cell lymphoma (COG ANHL 0131)

Phase	Drug	Day
Induction	Doxorubicin 75 mg/m²	1, 22
	Prednisone 40 mg/m²	1–28
	Vincristine 1.5 mg/m²	1, 8, 15, 22, 29
	IT methotrexate	1, 8, 22
Maintenance[a] (every 21 days)	Doxorubicin 30 mg/m²	1[b]
	Methotrexate 60 mg/m²	1[b]
	Vincristine 1.5 mg/m²	1
	Prednisone 120 mg/m²	1–5
	6-Mercaptopurine 225 mg/m²	1–5
	IT methotrexate (cycles 1–3 only)	1

Adapted from Alexander S, Kraveka JM, Weitzman S, Lowe E, Smith L, Lynch JC, et al. Advanced stage anaplastic large cell lymphoma in children and adolescents: results of ANHL0131, a randomized phase III trial of APO versus a modified regimen with vinblastine: a report from the children's oncology group. Pediatr Blood Cancer 2014 Dec;61(12):2236–2242 [104]

[a]Maintenance cycles given every 21 days for a total therapy duration of 1 year

[b]Methotrexate substituted for doxorubicin when the cumulative dose of doxorubicin reached 300 mg/m²

and cytarabine or the replacement of vincristine with vinblastine did not significantly improve outcomes (Table 46.8).

The BFM group during the same time period conducted trials which included patients with ALCL evaluating short course, "pulse therapy" as was used for B-NHL patients with initial therapy duration of 2–5 months based on stage (Table 46.9). The ALCL99 backbone of therapy was adapted from the BFM regimen. It includes dexamethasone, cyclophosphamide, ifosfamide, etoposide, doxorubicin methotrexate cytarabine, prednisone, and vincristine with 6 cycles given over 5 months for all except stage I patients. Cumulative doses of each agent are relatively low and therefore associated with lower long-term risks. The regimen does require inpatient care and is associated with substantial short morbidity including relatively high rates of febrile neutropenia and mucositis. ALCL-99 randomized patients to 1 gm/m² MTX over 24 h vs. 3 gm/m² over 3 h and found equal efficacy but less toxicity with the shorter infusion. The 3 gm/m² over 3 h is thus the current standard [103].

Vinblastine as a single agent has been known to have activity and in some patients be curative in the setting of relapsed disease [107, 108]. As a result, the European consortium study protocol ALCL-99 as well as the COG ANHL0131 protocol were designed to test the addition of weekly vinblastine to the BFM and APO backbones, respectively, in newly diagnosed patients. In both of these trials there was no statistically significant improvement in outcome with the addition of vinblastine.

Table 46.9 Outcome for patients with anaplastic large-cell lymphoma

Protocol	Number	Stage or risk group	Outcome (%)	References
Localized disease				
POG 9219	58	I/II	84% 5-year EFS	Link et al. [85]
BFM-90	8	I	100% 5-year EFS	Seidemann et al. [101]
	20	II	79% 5-year EFS	
Advanced disease				
BFM-90	55	III	74% 5-year EFS	Seidemann et al. [101]
	6	IV	50% 5-year EFS	
CCG 5941	86	III/IV	68% 5-year EFS	Lowe et al. [105]
			80% 5-year OS	
POG 9315	86	III/IV	72% 4-year EFS	Laver et al. [84]
			88% 4-year OS	
ALCL-99	352	I/II (90), III/IV (262)	74% 2-year EFS	Brugieres et al., Le Deley et al. [95, 106]
			93% 2-year OS	
COG 0131	125	III/IV	74% 3-year EFS	Alexander et al. [104]
			84% 3-year OS	

Several factors have been found to be prognostic in children with ALCL. Patients with disease in their mediastinum, visceral disease (defined as lung, liver, or spleen), or skin involvement had a poorer outcome in multivariate analysis [109]. By histology, lymphohistiocytic and small cell variant subtypes are associated with worse outcomes [104, 110].

In the context of patients treated with BFM type therapy the most powerful prognostic data comes from biological markers. The detection of *NPM-ALK* fusion gene transcript by quantitative PCR from blood and morphologically negative bone marrow at the time of initial diagnosis (minimal disseminated disease, MDD) was highly prognostically significant [24]. In addition, persistence of measurable residual disease (MRD) by *NPM-ALK* PCR in the blood following the first course of chemotherapy is associated with a very high risk of disease recurrence [111]. An immunological response at diagnosis has also been shown to be of prognostic significance in ALCL. Antibodies to ALK protein were detected in 95% of all ALK-positive patients at diagnosis. A low antibody titer was associated with advanced-stage, clinical features of mediastinal and visceral organ involvement, male gender and high numbers of

circulating tumor cells by PCR. In addition, antibody levels correlated significantly with the risk of relapse [112].

When considered together, the combination of MDD at diagnosis measured by quantitative PCR and the ALK antibody titer was found to be highly predictive of outcome in a retrospective evaluation of 128 patients from the BFM and Italian groups. In this study high-risk patients (MDD positive and ALK antibody low) had a progression-free survival of 28% compared to those who were low risk (MDD negative and ALK antibody high) of 93% and intermediate-risk group with a PFS of 68% [113].

There are a number of targeted therapies for children with ALCL currently being investigated in clinical trials that may alter the therapeutic landscape. Brentuximab vedotin is an anti-CD30 antibody linked to an antimicrotubule agent, monomethyl auristatin E. In heavily treated adult patients with relapsed/refractory ALCL 57% achieved a CR in a phase 2 trial [114]. As a single agent it is generally well tolerated though with neuropathy in 40% of patients. The use of this agent in newly diagnosed patients together with standard combination chemotherapy is being evaluated in ongoing adult and pediatric studies [115].

ALK inhibitors are very promising agents for the treatment of children with ALK+ ALCL. In a phase I trial which included children with relapsed/refractory ALCL, 7 of 8 patients achieved a CR [116]. Similarly in a retrospective adult trial 9 of 9 patients with refractory ALCL achieved a CR. Unlike adult lung cancer, responses have been durable in some of these patients continuing with single agent therapy [117]. The optimal duration of therapy is unknown. Trials evaluating the safety and utility of adding crizotinib to multiagent chemotherapy for newly diagnosed patients are being conducted through the COG and are being planned by the European pediatric EICNHL group. There are numerous second generation ALK inhibitors in development. Strategies to best evaluate these agents in children with this relatively rare lymphoma will require careful design and cooperation.

Summary of the Findings of the Cooperative Group Trials

1. Children with ALCL treated on different protocols have a similar outcome with an EFS of approximately 75%.
2. Despite the proven efficacy in patients with relapsed disease, the addition of weekly vinblastine to upfront therapy has not been shown to improve outcomes.
3. Patients with ALCL who develop relapse have high response rates to salvage chemotherapy, though the optimal curative therapy in this setting remains unclear.
4. Novel therapies, including monoclonal antibody-drug conjugates and ALK inhibitors, are likely to be important tools in the care of patients with ALCL.

Treatment of Relapsed Disease

Burkitt Lymphoma and Large B-Cell Lymphoma

Children who relapse following modern intensive protocols are difficult to cure. Relapses in BL and DLBCL are uncommon, but usually occur in the first year following therapy and carry a dismal prognosis. A UK-based multicenter study showed less than 30% survival in patients with relapsed BL and DLBCL [118]. Similarly, across three LMB studies, LMB89, FAB/LMB96, and LMB2001, 5% of patients with B-NHL relapsed at a median of 4.8 months for BL and 22 months for DLBCL and PMBCL and had an overall survival of 30% at 6 years [119]. Prognostic factors predicting better survival included Group A or B with LDH <2× ULN, large cell histology, relapse after 6 months, and relapse at only 1 site.

Since an initial response is usually required before referral for HSCT, novel therapies added to chemotherapy are being tested for B-NHL. The use of monoclonal antibodies (MAB) targeted to specific antigens on the tumor cell, for example, has produced compelling results for the treatment of adults with NHL. Reports have included a variety of antibodies, unlinked or linked to toxins such as ricin or to radioisotopes such as tositumomab (iodine-131 labelled anti-CD20 antibody) and ibritumomab (yttrium-90 labelled anti-CD20 antibody) [120, 121]. Radioimmunotherapy with tositumomab was shown to be effective in myeloablative regimens followed by autologous HSCT among adults with relapsed B-cell NHL, resulting in a 3-year OS of 59% and progression-free survival (PFS) of 51% [120]. It has also been demonstrated that adequate stem-cell collections are possible following MAB therapy even when the antibodies are linked to iodine-131 or yttrium-90.

In children with recurrent/refractory B-cell NHL, CYVE chemotherapy was effective in two-thirds of patients initially treated with Group A or B LMB therapy [119]. In a COG prospective phase II study, the addition of rituximab to ifosfamide, carboplatin and etoposide (ICE) chemotherapy achieved a complete plus partial response rate of 60% in 20 patients with BL, B-ALL, and DLBCL [122]. Following salvage chemotherapy, patients proceed to high-dose chemotherapy with autologous stem cell transplantation for consolidation therapy. There was zero percent chance of survival for non-responding patients.

Lymphoblastic Lymphoma

With the current intensive protocols, relapse or refractory disease is seen in about 15–20% of patients with LBL. Relapse occurs most commonly in the mediastinum but may be systemic and the outcome is poor with cure rates of between 15

and 30% [123, 124]. In general, the same salvage protocols used for T-ALL are tried but particularly in T-LBL the chance of obtaining a CR is low and the results of trials with combinations of chemotherapy with new agents such as nelarabine/etoposide/cyclo (NECTAR), carfilzomib plus 4 drug induction and mTOR or NOTCH1 inhibitors are awaited.

In B-LBL new agents that have been shown to be effective in B-lineage ALL may prove to be useful, including anti-CD20 antibody for those who are CD20 positive and the bispecific CD19/CD3 antibody Blinatumomab for the majority of B-LBL who are CD19 positive.

In those patients who do achieve a CR or VGPR, allogeneic transplant is indicated (see below).

Anaplastic Large Cell Lymphoma

Unlike the other lymphomas, ALCL often remains chemosensitive at relapse. The outcome for patients with relapsed ALCL is more favorable than for other NHL types in children with chances of survival approaching 60% [107, 125]. Prognostic features at relapse include time to relapse and CD3 positivity of tumor cells at the time of initial diagnosis. The EICNHL relapsed ALCL protocol stratified patients into risk groups with higher risk patients achieving 3 year EFS of 64% with allocation to allogeneic stem cell transplant, whereas those in the intermediate group treated with autologous transplant had a less good outcome with a 3 year EFS of 41%. The low-risk group was treated with 2 years of weekly vinblastine and has very favorable outcomes with a 3 year EFS of 85% [126].

As discussed above, ALK inhibitors are very promising agents for the treatment of children with ALK+ ALCL and may be valuable for therapy of relapsed/refractory disease. The development of second and third generation ALK inhibitors may prove to be useful in children whose disease recurs post therapy that included crizotinib. The optimal duration of ALK inhibitor therapy is unknown.

The use of novel agents in addition to conventional therapy including stem cell transplant for patients with relapsed and refractory ALCL requires ongoing investigation.

Hematopoietic Stem Cell Transplantation

The value of autologous vs. allogeneic transplant remains controversial and appears to depend on the NHL subtype. A review of data from the Center for International Bone Marrow Transplant Research (CIBMTR) from 1990 to 2005 showed that 5-year EFS for allogeneic HSCT was equal to autologous transplants for patients with DLBCL (50% vs. 52%, respectively), Burkitt lymphoma (31% vs. 27%, respectively), and ALCL (46% vs. 35%, respectively). Only for lymphoblastic lymphoma did allogeneic HSCT demonstrate an advantage compared to autologous transplantation (40% vs. 4%, respectively, $P < 0.01$) [127]. This is in agreement with an earlier study in which the advantage of allogeneic over autologous transplantation for LBL showed a lower relapse rate but at the expense of higher treatment-related mortality [128], as well as BFM data in which the only patients with relapsed T-LBL to survive were those who receive an allogeneic HSCT [123]. A long-term single institution study of pediatric patients with NHL demonstrated an OS and DFS of 55% and 53%, respectively, at a median follow-up of 9.7 years. The most important prognosticator for survival was response to salvage chemotherapy with DFS of 61% for chemotherapy-sensitive disease compared to 25% for nonresponders. The study found a 50% DFS and OS for 11 patients with LBL who received an allogeneic HSCT, with a median follow-up of 12 years [129]. One of the study recommendations was autologous SCT if no matched donor, related or unrelated, was available, but given the recent improvements in results using haploidentical donors, with current knowledge all relapsed LBL patients should be referred for allogeneic SCT.

For relapsed ALCL, the BFM group has used allogeneic transplant with conditioning regimens that include total body irradiation, etoposide, and cyclophosphamide, resulting in a 3-year EFS of 75% [130]. Many of the patients in this trial had failed multiple protocols and all 5 who had failed previous autologous transplantation survived following allogeneic HSCT.

Another potentially important approach involves post-transplantation therapy directed at minimal residual disease, such as approaches aimed at increasing the host immune response to residual lymphoma cells, or using novel targeted anti-lymphoma therapies. Several studies in children with relapsed B-cell NHL have demonstrated that anti-CD20 monoclonal antibody is well tolerated when given after transplantation [131].

In summary, HSCT offers an OS of around 50% for patients with relapsed NHL. The decision regarding autologous vs. allogeneic SCT should be based first on histology as well as risk factors, at least in ALCL. As supportive care measures improve, the balance may swing towards allogeneic SCT given the proven graft vs. lymphoma effect.

For B-NHL, most of whom are CD19 positive irrespective of histology, chimeric antigen receptor (CAR) T-cell immunotherapy with specificity for CD19 has shown major promise for the most treatment resistant pediatric and adult B-cell acute leukemias, even those that have failed HSCT [132, 133]. Second generation CAR T-cells for treatment of adult relapsed B-NHL has recently been reported in a phase I study with 75% 1-year PFS and without any reported cytokine release syndrome usually seen in ALL patients [134]. Further studies are needed to confirm this data.

Table 46.10 Summary characteristics of the four major pediatric non-Hodgkin lymphomas

	Burkitt lymphoma (sporadic type)	Lymphoblastic Lymphoma	Diffuse large B-cell lymphoma	Anaplastic large-cell lymphoma
Immunophenotype	Mature B-cell	Precursor T-cell (80%)	Mature B-cell	Mature T-cell (80%)
		Precursor B-cell (20%)		Null-cell (20%)
Genetics	t(8;14) in 80%	t(10;14)	Bcl-6 (30%)	t(2;5) seen in 90%
	t(8;22)	t(1;14)		t(1;2)
	t(2;8)	t(11;14)		inv 2
	13q deletions			
Site	Abdominal (90%)	T-LL: mediastinum, supradiaphragm	Abdomen, mediastinum, any node	Nodes, skin, bone, lung
	Nasopharynx (10%)	B-LL: unusual sites		
Spread	Bone marrow ++	Bone marrow ++	BM uncommon	BM uncommon
	CNS ++	CNS +	CNS uncommon	CNS uncommon
Event-free survival[a]				
Localized (%)	95	92	93	90
Advanced (%)	90	80–85	90	70–80

[a]Collective results of the cooperative study groups

Conclusion

The survival of children with NHL of all four subtypes (Table 46.10) has dramatically improved, so that the majority of pediatric patients with lymphoma can now be cured. However, several important challenges remain. These include reduction of treatment-related toxicity without sacrificing cure rates, identification of new prognostic factors that will allow therapy to be tailored to specific risk groups, and development of effective treatment for patients who have resistant disease despite modern intensive protocols.

It is unlikely that continued intensification of chemotherapy will achieve further major improvement in responses, although modifications currently being evaluated may improve survival by decreasing therapy-related toxicity. The introduction of therapies targeted to specific molecules on the malignant cell surface membrane or engineered T-cell adoptive immunotherapy has added an exciting new dimension to future therapeutic strategies.

Finally, the importance of coordinated international collaborative efforts to facilitate the development of more effective therapies aimed at tumor-specific targets cannot be overemphasized.

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Lymphoma in Other Diseases

47

Jennifer Crombie and Dan L. Longo

Introduction

In the United States in 2016, non-Hodgkin's lymphoma (NHL) comprised 4.3% of all new cancers and 3.4% of cancer related deaths [1]. The incidence of NHL has increased considerably since the early 1970s, though has stabilized in the last 10 years. While the cause of lymphoma is unexplained in the majority of cases, lymphoma is associated with various diseases and their treatments. For example, patients with the human immunodeficiency virus (HIV) have a 60–200-fold risk of developing NHL [2, 3]. Other viruses, such as Epstein-Barr virus (EBV) and HTLV-1, and even certain bacteria, have been associated with the development of lymphoma as well. Patients who have undergone solid organ or bone marrow transplantation have an increased risk of lymphoma, particularly in the setting of iatrogenic immune suppression. Autoimmune disease and the therapies used in their management have also been linked to malignancy. Immune dysregulation, as is seen in patients with genetic immunodeficiencies, also results an increased risk of lymphoma compared to the general population.

Lymphomas that occur in the context of other diseases often have distinct histologic, morphologic, and clinical presentations and require unique therapeutic considerations that will be further addressed here. The vast majority of secondary lymphomas are of B-cell origin. They tend to be aggressive histologically as well as in advanced stage at presentation. Extranodal sites are affected in a high proportion of cases, particularly the bone marrow, central nervous system (CNS), and GI tract. These lymphomas often occur in association with EBV infection and other viruses.

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The development of lymphoma in these cases typically results from a multistep process in which a hierarchy of events impinges on the normal control of cell growth. The series of biologic changes that lead to cancer involves the accumulation of discrete genetic alterations that provide the basis for the transition from normal to premalignant to malignant lesions. Although details regarding mechanisms of lymphomagenesis remain to be clarified, three key components appear critical: (1) polyclonal proliferation of B cells; (2) the benign, or premalignant, but extensive B-cell hyperplasia that increases the chances of acquiring critical genetic alterations; (3) the inherent immune deficiency leads to a failure of immune surveillance, allowing the abnormal cell growth of occasional clones to proceed unchecked. The result is ultimately a monoclonal or oligoclonal cellular proliferation that constitutes the observed lymphoma.

Throughout the remainder of this chapter, the ways in which immune dysregulation is thought to predispose to lymphoma as well as the specific clinical presentations and treatment considerations will be further explored.

Infectious Agents in the Etiology of Lymphomas

Epstein-Barr Virus (EBV)

EBV is a transforming DNA virus of the herpesvirus family that targets oro- and nasopharyngeal epithelial cells and human B lymphocytes [4]. Thus, as will be discussed later, EBV appears to be involved in lymphomagenesis in the settings of AIDS, iatrogenic immunosuppression, and inherited immunodeficiency diseases such as X-linked lymphoproliferative disease (LPD) and ataxia-telangiectasia. EBV infection also plays a role in the development of the endemic form of Burkitt's lymphoma, which is primarily seen in children in Africa, as well as a range of malignancies involving B cells, T cells, and natural killer (NK) cells [5].

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EBV is shed through oral secretions of infected hosts and is thought to infect up to 90% of the population [6]. Primary infection is usually asymptomatic, though can also result in infectious mononucleosis, a clinical syndrome characterized by fever, pharyngitis, lymphadenopathy, and fatigue. It is thought that the virus initially targets B cells located in oropharyngeal epithelium and mucosa, though ultimately inhabits memory B cells, where it remains as a lifelong inhabitant of the host [7, 8]. The virus is intermittently reactivated in the oropharyngeal epithelium allowing for transmission.

EBV Biology

The EBV viral genome is encased within a nucleocapsid that is surrounded by the viral envelope. The major envelope glycoprotein, gp350, binds to the viral receptor, the CD21 molecule, which is closely related to the receptor for the C3d component of complement [9, 10]. This receptor is present on the surface of epithelial cells, B cells and some T cells [9, 11]. The major-histocompatibility-complex (MHC) class II molecule serves as a cofactor, thus allowing for infection of cells [12].

The EBV genome consists of a linear DNA molecule that encodes nearly 100 viral proteins. These proteins in turn regulate the expression of viral genes, replication of viral DNA, formation of the virion, and modulation of the host immune system. After infecting B cells, the linear EBV genome becomes circular, forming an episome. In this form, the virus remains as a lifelong latent infection.

Of the genes that make up the EBV genome, only specific subsets are expressed during the latent infection of EBV-immortalized cells. These include six EBV nuclear antigens [EBNA-1, -2, -3A, -3B, -3C, and leader protein (LP)], three latent membrane proteins (LMP-1, -2A, and -2B), and two abundant non-polyadenylated, nontranslated small RNAs [EBV-encoded small RNA (EBER)-1 and EBER-2]. Six of these (EBNA-1, -2, -3A, and -3C; LMP-1; and LP) are required for immortalization, whereas two (LMP-2A and -2B) are believed to maintain EBV latency.

At least three types of latency have been demonstrated, each of which is characterized by a specific array of latent gene expression. Furthermore, the different histological types of EBV-related lymphomas tend to fall into specific latency types [13]. EBNA-1 is expressed in all the latency types and is the only viral protein expressed in type I latency (Lat I). Burkitt's lymphoma, whether endemic or AIDS-related, generally displays a Lat I phenotype [14]. Type II latency (Lat II) includes expression of the LMPs in addition to the ubiquitously expressed EBNA-1. The Lat II subtype has been described in a subset of Burkitt's lymphomas as well as in Hodgkin's disease, nasopharyngeal carcinoma, and T-cell tumors [15]. Lastly, type III latency (Lat III) involves expression of all the latent viral proteins. The two small RNAs (EBER-1 and EBER-2) are expressed in all three latency types. AIDS-related large-cell and immunoblastic lymphomas have been shown to fall into all three latency categories.

Following initial infection, EBV primarily resides in the long-lived memory B cells. There are two proposed mechanisms in which the virus inhabits these cells [8]. The first is known as the germinal center model, in which EBV-infected memory B cells are generated through normal B-cell maturation involving germinal centers. Infection of naïve B cells leads to the expansion of cells expressing type III latency proteins [8]. The cells enter the germinal center where they express latency II proteins and ultimately exit as memory B cells. EBV-infected memory B cells downregulate latent gene expression and enter what is known as latency 0, allowing the cells to avoid recognition by the immune system. The cells switch on the EBNA-1 protein when required for cell division during latency 1 and differentiate into plasma cells in order to reactivate the EBV lytic cycle.

The alternative model, known as the direct infection model, suggests that germinal center B cells or memory B cells are directly infected by EBV. In these cases, EBV is able to form a memory phenotype while bypassing the requirement of a germinal center reaction and somatic hypermutation [8].

Two distinct types of EBV isolates, type 1 and type 2, have been described, each of which is subdivided into multiple strains [16]. These strains are distinguished by polymorphisms in the latent genes encoding the nuclear antigens EBNA-2, -3A, -3B, and -3C, and the EBER genes. EBV type 1 has a greater transforming capacity and is the predominant type (90%) in lymphocytes of EBV-infected individuals. While the majority of people harbor a single virus strain, multiple strains have been seen, especially in immunocompromised hosts. For example, EBV type 2, which is relatively rare in the healthy Caucasian population, is observed in over 30% of HIV-positive men who have sex with men (MSM) and in 10% of HIV-infected hemophiliac individuals [16].

Individual EBV type 1 strains do not appear to be restricted to specific EBV-associated diseases or tumor types. While certain polymorphisms have been associated with specific clinical presentations, these associations are believed to reflect the most prevalent circulating EBV strains in the population, rather than the disease presentation. For example, the 3′-terminal of the LMP-1 gene has a characteristic 30-base pair deletion in nasal T/NK-cell lymphomas in a Chinese population and a 69-base pair deletion in Burkitt's lymphoma of Turkish children [17, 18]. This view is supported by the appearance of identical variant strains in other EBV-associated diseases, such as nasopharyngeal carcinoma and peripheral T-cell lymphomas, as well as in healthy virus carriers in the same populations. Specific EBV antigen polymorphisms have been shown to correlate with oncogenic potential in cell culture, though has not been substantiated in vivo [19].

Accumulation of Genetic Lesions

The contribution of the EBV antigens to cellular transformation is complex. EBNA-1, a phosphoprotein that binds to both viral DNA and RNA, maintains the EBV genome as an extrachromosomal plasmid in immortalized cells [20]. EBNA-2 counters transcriptional repression by interacting with the inhibitory transcription factor CBF1/RBP-Jκ [21]. EBNA-2 also activates EBNA-3A, which is essential for initiation of B-cell immortalization. EBNA-3C, which is also necessary for immortalization, has been shown to activate the promoter of the B-*MYB* oncogene, leading to cell cycle progression. EBNA-3C may promote lymphogenesis through interaction with the *RAS* oncogene as well [22]. The products of specific EBV genes may also exert more direct effects on oncogene expression. Even in the absence of a c-*MYC* translocation, the presence of the EBV genome in the Burkitt's lymphoma cells is reversibly associated with increased levels of c-*MYC* messenger RNA, due to stabilization of the c-*MYC* transcript in the late exponential phase of growth [23]. In addition, EBV early protein EB2 has the ability to transform cells, possibly by regulating c-*MYC* expression at a posttranscriptional level [24].

EBV is also thought to promote transformation through expression of LMP-1, which is transcriptionally regulated by EBNA-2 [25]. LMP-1 activates multiple signaling pathways, including mitogen-activated protein kinase (MAPK), c-Jun N-terminal kinase (JNK), phosphatidylinositol 3-kinase (PI3K)/Akt, and NF-κB [26]. LMP-1 also induces transcription of many genes to affect apoptosis, cell cycle progression, cell proliferation, metastasis, and angiogenesis [26, 27]. An association between specific mutational hot spots in the EBV *BNLF*-1 gene that encodes the LMP-1 protein and various lymphoproliferative disorders (e.g., Hodgkin's disease, angioimmunoblastic lymphadenopathy (AILD), B-immunoblastic lymphoma, and peripheral T-cell lymphoma) have also been demonstrated. Similarly, reduction in transcriptional expression of LMP-1 by triptolide in vitro was also found to decrease proliferation of EBV-positive B-cell lymphoma cells [27]. A meta-analysis has also shown that LMP-1 expression appears to be an unfavorable prognostic factor for overall survival for non-Hodgkin lymphoma in Chinese patients [28].

Overall, it is thought that EBV likely provides the initiating insult in the multistep process of lymphomagenesis by increasing growth of affected cells. The ability of EBV to infect cells latently and to immortalize them also leads to expansion of the affected B-lymphocyte population. The ability of the virus to evade the immune system and avoid apoptosis further provides a survival advantage.

Host Response

The normal host displays cellular mechanisms that limit the number of virally transformed B lymphocytes. Healthy, immunocompetent EBV-seropositive individuals maintain a vigorous cytotoxic T-cell response against EBV latency antigens (EBNA-2, -3A, -3B, or -3C). Patients with infectious mononucleosis have cytotoxic T lymphocytes (CTLs) that can lyse EBV-transformed B-cell targets in culture, implying that such cells play an active role in eliminating the infected B lymphocytes in vivo [29]. In severely immunocompromised individuals, however, a deficit of appropriately targeted antigen-specific CTLs may allow EBV-infected B-cell proliferation to go unchecked. This could explain the finding that many posttransplant lymphoproliferative disorders are positive for EBV within tumor cells. Adoptive immunotherapy with healthy donor-derived EBV-specific CTLs modified with specific target EBV antigens has been explored in an effort to reconstitute cell-mediated immunity against EBV infection and thereby to prevent EBV lymphomas in immunocompromised patients [30, 31].

While a deficit of immune surveillance may explain the escape of EBV-transformed cells in immunocompromised patients, in immunocompetent individuals, malignant cells also evade immune control by failing to express virus-induced surface antigens that normally serve as targets for CTLs. EBV-related tumors arising in healthy individuals (including EBV-related Burkitt's lymphoma, Hodgkin's disease, and nasopharyngeal carcinoma) fail to express the majority of EBV latency antigens, in contrast to the viral antigen expression that has been noted in some posttransplantation and AIDS-associated lymphomas. Specifically, CTL responses against LYDMA, the virus-induced lymphocyte-detected membrane antigen, limit EBV-infected B-cell numbers in healthy carriers [32]. In virus-associated Burkitt's lymphomas, the malignant clone is frequently insensitive to such surveillance by effector CTLs derived from healthy virus-immune donors, suggesting that in these cases the malignant cells do not express LYDMA.

In addition to CTLs, EBV can be opposed by interferon-α and -γ, other cytokines, natural killer cells, and antibody-dependent cellular cytotoxicity. Immunologic dysfunction involving these mechanisms can similarly lead to failure of adequate surveillance and control of EBV-induced lymphocyte proliferation. For instance, the EBV-encoded protein BCRF-1 is structurally and functionally homologous to the cytokine IL-10, or cytokine synthesis inhibitory factor (CSIF). BCRF-1 suppresses T cell and perhaps natural killer cell synthesis of interferon-γ and IL-2, resulting in what amounts to functional suppression of T-cell antiviral activity [33].

Other immunologic abnormalities that may impair elimination of virally transformed lymphocytes include upregulation of autocrine factors such as IL-6, a cytokine that stimulates B-cell growth, and soluble CD23, the receptor that promotes immortalization in response to IL-1.

It has also been shown that HLA class I type can impact the immune surveillance of EBV by CD8 + T cells. In western

European populations, HLA-A*01 and HLA-B*37 are associated with an increased susceptibility to EBV, while HLA-A*02 is protective [34, 35]. Specifically, HLA-A*02 seems to mediate a CD8+ T-cell response against latency II antigens that may reduce the incidence of malignant disease [15].

Clinical Features of EBV-Associated Lymphomas

Burkitt's Lymphoma

The prototype of the EBV-related lymphomas is Burkitt's lymphoma. In the endemic (African) form of the disease, the EBV genome is detected in 98% of cases, in contrast to the sporadic form of Burkitt's lymphoma, in which only 15–20% of cases are EBV positive [7, 36]. Both endemic and sporadic Burkitt's lymphoma cells carry a translocation involving chromosome 8q24, which harbors the c-*MYC* gene, and one of the Ig loci. As a result of this translocation, the oncogenic c-*MYC* is upregulated, contributing to transformation of the affected cell. In the majority of cases, the reciprocal translocation exists between chromosome 8q24 and chromosome 14q32, the locus of the Ig heavy chain (IgH) gene, though translocations of chromosome 8q24 to chromosome 22q11 (the Ig-λ locus) and chromosomal 8q24 and chromosome 2q24 (the Ig-κ locus) have also been seen [37]. Although a cytogenetic translocation is demonstrable in all cases of Burkitt's lymphoma, the actual breakpoint on chromosome 8 may be outside the c-*MYC* locus, as is typical for the endemic form of Burkitt's lymphoma [38]. By contrast, sporadic Burkitt's lymphoma generally harbors multiple breakpoints within or close to the 5′-region of c-*MYC* (in the noncoding first exon or the first intron) [38].

Both types of Burkitt's lymphoma result in a histologically similar tumor with round monomorphic cells and surrounding macrophages, creating what is often called a "starry sky appearance" [36]. The endemic form, however, has a unique phenotype typically with the formation of tumors in the jaw or abdomen of children. In these cases, there may be a correlation between malaria infection, as they most commonly occur in areas of equatorial Africa and Papua New Guinea, where malaria is holoendemic. In these regions, asymptomatic EBV infection in children is common. A subsequent acute malarial infection can lead to dysregulation of the immune system and reactivation of the latent virus. EBV-induced polyclonal B-cell proliferation ensues in the absence of a complete T-cell response, allowing for the characteristic 8;14 translocation and dysregulated cell growth.

The surface phenotypes for both the sporadic and endemic forms of Burkitt's are similar (CD10+, CD77+, CD23-, BCL6+, and PAX5+) and are consistent with the likely precursor being memory B cells for endemic and germinal center B cells for sporadic forms of the disease [39]. In endemic Burkitt's lymphoma, the c-*MYC* gene is typically translocated unrearranged into a breakpoint in the V_H-D-J_H recombination region, generally into the J_H segment [37]. This type of translocation appears to result from aberrant functioning of the recombinase enzyme that under normal conditions is responsible for routine Ig gene rearrangement in memory B cells. In the case of sporadic Burkitt's lymphoma, the first exon of the disrupted c-*MYC* gene is sometimes translocated, in inverted orientation, into a breakpoint in the switch region of the heavy chain gene (Sμ) [40]. The presumed error in this case is on the part of isotype switching enzymes that are normally active in more mature B cells that have the capacity to secrete Igs.

While *MYC* overexpression results in increased proliferation, mutated cells must also overcome increased sensitivity to apoptosis signals. Accordingly, approximately 30% of endemic, EBV+ Burkitt's lymphomas carry p53 mutations, and those with wild-type p53 frequently overexpress the p53 antagonist MDM2 [41]. Over the past decade an increased understanding of a second MYC-activated pathway to apoptosis that involves the apoptotic activator of the Bcl-2 family, BIM, has also been described. BIM initiates apoptosis by binding and inactivating pro-survival BCL2 family members (including BCL2 and MCL1) and by activating apoptotic effectors, BAX and BAK [42]. Thus, dysregulated MYC expression can trigger apoptosis through multiple and independent pathways (p14ARF, 19ARF, p53, or BIM) and inactivation of any of these can allow proliferation and lymphoma development.

EBV-Associated Hodgkin's Lymphoma

EBV was first noted to have an association with Hodgkin's lymphoma in epidemiologic studies. A threefold increase in the relative risk of developing Hodgkin's lymphoma was also observed following infectious mononucleosis [43]. This association was further supported by data showing that a prior history of infectious mononucleosis was more likely to correlate with EBV-positive than with EBV-negative Hodgkin's lymphoma [44]. Furthermore, elevated levels of IgG and IgA antibodies against EBV capsid antigen, EBNA, and early antigen D have been seen in individuals who subsequently developed Hodgkin's lymphoma [45]. Episomal, clonal EBV DNA has also been detected in involved lymph nodes of Hodgkin's lymphoma patients, where it is localized to Reed–Sternberg and variant cells [46]. The Reed–Sternberg cells of Hodgkin's lymphoma exhibit a Lat II pattern, in which latent gene expression is restricted to EBNA-1, LMP-1, and LMP-2, differing from the latency type seen in other EBV-associated lymphomas (e.g., Burkitt's lymphoma) but resembling that seen in nasopharyngeal carcinoma [46, 47].

A higher expression of proliferating cell nuclear antigen (PCNA) has been noted in EBV-positive than EBV-negative Hodgkin's lymphoma, suggesting that EBV probably promotes the cell cycling of Reed–Sternberg cells [48]. Interestingly, higher PCNA expression in a tumor appears to confer a survival advantage to the patient.

EBV-specific CTLs have been demonstrated among the tumor infiltrating lymphocytes from Hodgkin's lymphoma biopsies, indicating an ongoing immune response against EBV-carrying cells [49]. Despite this observation, EBV-associated Hodgkin's lymphoma tumor cells have evolved immunologic strategies to evade such ongoing cytotoxic responses. In one study, the cytokine IL-10, an inhibitor of cell-mediated immunity, was shown to be upregulated by the EBV antigen LMP-1 in tumor cells of Hodgkin's lymphoma [50]. EBV also increases the expression of programmed death1 (PD-1) ligands, which when bound to the PD-1 receptor immune cells, can serve as a checkpoint to limit T-cell-mediated responses [51, 52].

Mixed cellularity Hodgkin's lymphoma, commonly found in children and older adults and in developing countries, is the subtype most likely to be associated with EBV (up to 90% of cases). Lymphocyte-depleted tumors are also frequently EBV positive, whereas 30% of nodular sclerosing and only rare cases of the lymphocyte-predominant subtype carry the virus [53]. Finally, epidemiologic studies have shown that 50–70% of cases in Western countries are positive for the EBV genome, with EBV-positive disease being found more often in males than females [54]. Although the evidence for a causal relationship between EBV infection and Hodgkin's lymphoma is not yet considered definitive, the accumulating data continue to strengthen the likelihood of such an association.

Diffuse Large B-Cell Lymphoma

Diffuse large B-cell lymphoma (DLBCL), which is one of the most frequent subtypes of non-Hodgkin's lymphoma in adults, can also be associated with EBV infection. EBV-positive DLBCL of the elderly, for example, has been incorporated into the 2008 World Health Organization classification as a provisional entity [55]. Patients with EBV+ monoclonal B-cell tumors must be greater than 50 years old and have no known evidence of immunodeficiency in order to meet criteria [56]. The malignant B cells are typically positive for the leukocyte common antigen CD45 and B-cell markers CD20, CD19, CD79a, and PAX-5 [57]. This malignancy is most commonly seen in Asian countries, but has been identified in a smaller fraction of Western countries as well. It is aggressive subtype of lymphoma and patients typically have a poorer prognosis as compared to patients with EBV-negative disease. EBV uses a type II latency pattern, as is seen in Hodgkin's lymphoma, or a type III pattern as is seen in post-transplant lymphoproliferative disorder (PTLD).

It has been postulated that EBV+ DLBCL of the elderly may result from the senescence of the immune system that is seen with aging [55]. Specifically, it is thought that B-cell diversity and the absolute number of T lymphocytes decreases [58, 59]. Studies have also implicated activation of the NF-kB signaling pathway by LMP-1 in the pathogenesis [58]. Interestingly, higher levels of EBV DNA in tumor specimens has been shown to confer worse progression free survival and overall survival as compared to tumors with lower quantities [60]. While a dampened immune system may contribute to the development of this disease in older patients, it has been shown that gene expression profiling, microRNA profiling and treatment outcome is similar in younger patients and this arbitrary age cutoff may not be necessary [56].

Other EBV-Associated Malignancies

In addition to Burkitt's lymphoma, Hodgkin's lymphoma, and DLBCL, EBV is thought to contribute to the development of other less common subtypes of lymphoma. EBV is associated with a variety of T-cell lymphomas. While EBV primarily infects B cells, it is also thought to rarely infect T cells and natural killer (NK) cells. EBV infection in seen in all cases of nasal NK-T-cell lymphoma, which is endemic in East Asia. Immunophenotypically, these malignant cells are CD3e and CD56 positive and are almost always infected with clonal, episomal EBV. Quantification of EBV in the plasma can also predict tumor load and confer a poorer prognosis in these patients [61]. T/NK cell lymphoproliferative disease, which is characterized by fever, lymphadenopathy, and splenomegaly in non-immunosuppressed patients, is similarly related to EBV infection and more commonly seen in East Asian countries [62].

Plasmablastic lymphoma (PBL) is a rare type of NHL that arises from B cells that are in transition to plasma cells known as plasmablasts. This is a rare aggressive lymphoma that primarily occurs in the context of immunosuppression with a predilection for the oral and nasal cavity. Infection with EBV in these patients can lead to surge in plasmablast levels and potentially predispose to malignant transformation [63]. Lymphomatoid granulomatosis is another EBV-driven lymphoproliferative disorder that presents in the form of bilateral pulmonary nodules in the lungs. This is an angiocentric and angiodestructive entity defined by the presence of large B cells that are positive for EBER [64]. In primary CNS lymphoma, occurring in both the absence and presence of obvious immunodeficiency, EBV DNA has been identified in the lymphoma but not in the adjacent normal brain tissue [65]. The presence of EBV has been noted in thyroid lymphomas, as well as lymphoepithelial and

non-lymphomatous malignancies of the salivary gland, thymus, tonsil, oral mucosa/hairy leukoplakia of the tongue, esophagus, lung, uterine cervix, and breast. The significance of the EBV findings in many of these malignancies, however, still remains questionable [54].

Acquired Immune Deficiency Syndrome/Human Immunodeficiency Virus (HIV)-Associated Lymphomas

HIV

HIV is a retrovirus that targets CD4-positive lymphocytes through interactions with CD4 and the chemokine receptors, CCR5 and CXCR4. Infection subsequently leads to a progressive reduction in the CD4 T-cell count as well as lifelong latent infection in resting memory T cells. In 2012 an estimated 35.3 million people were infected with HIV [66]. Through a variety of mechanisms, which will be discussed in greater detail, patients with HIV are at increased risk of some forms of malignancy. Approximately 40% of patients with HIV will develop cancer, the majority of which will be lymphoma [67].

In 1985, HIV-associated lymphoma was first incorporated into the Centers for Disease Control and Prevention's definition of AIDS [68]. Despite an improvement in morbidity and mortality with the advent of antiretroviral therapy, patients with HIV remain at risk of developing a variety of malignancies [3]. While there have been reductions in lymphomas associated with lower CD4 counts, such as primary CNS lymphoma and primary effusion lymphoma, other subtypes of lymphoma, including Burkitt's lymphoma and Hodgkin's lymphoma, persist despite improved immune function [69, 70].

Accumulation of Genetic Lesions

Following an initial insult, such as the HIV infection, successive genetic changes can be superimposed on the expanded lymphocyte population resulting in malignant transformation of one or more clones. There are a variety of proposed mechanisms to suggest why this occurs. One theory is that HIV infection leads to chronic antigenic stimulation and persistent immune activation [3]. Additionally, defective immune surveillance in the setting of a reduced CD4-positive lymphocyte count allows for possible expansion of abnormal clones. Support for this model comes from the observation that HIV-related lymphadenopathy often predates the development of lymphoma [71]. Multiple monoclonal B-cell expansions have been documented in biopsies of AIDS–non-Hodgkin's lymphomas, as well as in a significant fraction of lymph nodes with the ostensibly benign polyclonal hyperplasia [72]. The fact that up to 60% of the AIDS–non-Hodgkin's lymphomas but only 10–20% of lesions of AIDS-related benign lymphoid hyperplasia contain oligoclonal B-cell expansions suggests a progression along a spectrum of malignancy, reflecting the accumulation of genetic alterations (Table 47.1) that culminate in the dominance of specific B-cell clones [73]. By contrast, non-Hodgkin's

Table 47.1 Genetic alterations and viral infection in AIDS-related non-Hodgkin's lymphomas[a]

Lymphoma histology: B-cell non-Hodgkin's lymphomas	Lymphoma frequency (%)	Dominantly acting oncogenes					Tumor suppressor genes		Viruses	
		c-*MYC* (%)	*BCL*-6 (%)	*BCL*-1 (%)	*BCL*-2 (%)	*RAS* (%)	*p53*	*RB1*	EBV	HIV
Systemic AIDS–non-Hodgkin's lymphoma	81[b]									
AIDS–Burkitt's lymphoma	26[c]	100	0	0	0	15	60	0	30	0
AIDS–diffuse large B-cell lymphoma	74[c]									
AIDS–large-cell lymphoma		20	20	0	0	15	0	0	30	0
AIDS–large-cell immunoblastic plasmacytoid lymphoma		20	20	0	0	15	0	0	90	0
AIDS–anaplastic cell lymphoma		NA[d]	0	0	0	NA[d]	0	NA[d]	90	0
AIDS–primary central nervous system lymphoma	19[b]	NA[d]	NA[d]	0	0	NA[d]	0	NA[d]	100	0

[a]Data from Gaidano G, Dalla-Favera R. Molecular pathogenesis of AIDS-related lymphomas. *Adv Cancer Res*. 1995;67:113–53
[b]Frequency among AIDS–non-Hodgkin's lymphomas; derived from numbers of cases reported by Beral et al. [2]
[c]Frequency within systemic AIDS–non-Hodgkin's lymphomas; derived from numbers of cases reported by Beral et al. [2]
[d]*NA* not available

lymphomas in the immunocompetent population only infrequently show multiple clonal B-cell expansions [73].

Cytokine production in the setting of HIV infection also contributes to the production of these clonal populations. Interleukin (IL)-6 and IL-10 are two cytokine regulators of B-cell growth that have been shown to play a role. IL-6, also known as BSF-2, or B-cell stimulatory factor, is produced by a variety of cell types, including monocytes/macrophages, T lymphocytes, and fibroblasts and has been shown to promote the growth and terminal differentiation of B cells [74]. B cells from HIV-infected individuals constitutively secrete high levels of IL-6, which in turn, contributes to polyclonal B-cell stimulation and nonspecific hypergammaglobulinemia. IL-6 serum levels in AIDS patients may also predict the subsequent development of non-Hodgkin's lymphoma [75].

IL-10 also acts in an autocrine fashion as a potent stimulator of B cells. Its B-cell differentiating capability may enhance Ig secretion, contributing to the hypergammaglobulinemia. IL-10 is also thought to inhibit apoptosis, thereby promoting B-cell viability. Finally, IL-10 inhibits the production of IL-2 and the antiviral cytokine, interferon-γ, by T cells, leading to impairment of immunosurveillance.

Among the genetic alterations contributing to B-cell non-Hodgkin's lymphomas in AIDS is activation of the c-*MYC* oncogene by means of its rearrangement into the vicinity of Ig regulatory elements (Table 47.1). All AIDS–small noncleaved cell lymphomas contain t(8;14) or t(8;22) translocations, resembling those found in Burkitt's lymphoma in the non-AIDs population. Single c-*MYC* rearrangements of this nature have been identified less frequently in AIDS–non-Hodgkin's lymphomas of other histologic types as well. At the molecular level, the t(8;14) translocations in AIDS–non-Hodgkin's lymphomas generally resemble the sporadic Burkitt's lymphomas, by involvement of the Sμ region of the IgH gene [76]. Mutations within the first intron as well as the coding region have also been demonstrated in AIDS–non-Hodgkin's lymphomas, primarily in Burkitt's and Burkitt's-like lymphomas [77].

In addition to c-*MYC*, translocations in AIDS–non-Hodgkin's lymphomas have been shown to involve the *BCL*-6 gene (Table 47.1) [73, 78]. *BCL*-6, which is located on chromosome 3q27 and codes for a zinc finger transcription factor, is truncated by virtue of its translocation in 30–40% of DLCL in immunocompetent hosts [73, 79]. Similarly, *BCL*-6 rearrangements in AIDS–non-Hodgkin's lymphomas are restricted to DLCL (occurring in 19% of AIDS–immunoblastic lymphomas and 25% of AIDS–large-cell lymphomas), never being observed in AIDS–Burkitt's or Burkitt's-like lymphomas. *BCL*-6 rearrangements are seen in both EBV-positive and EBV-negative lymphomas. Rearrangements involving c-*MYC* and *BCL*-6 have never been observed in the same biopsy specimen, suggesting that the two genetic lesions contribute to independent pathways to tumorigenesis [73].

The *BCL*-2 gene, whose protein product blocks cellular apoptosis, is rearranged in most low-grade follicular B-cell lymphomas and a smaller percentage of the more aggressive lymphomas. However, no rearrangement or mutation in this gene has been detected in AIDS–non-Hodgkin's lymphomas [80]. Similarly, no alteration has been demonstrated for *PRAD*-1/*BCL*-1, a gene that encodes cyclin D, an active component of the cell cycle machinery, whose translocation from chromosome 11q13 to chromosome 14q32 is associated with mantle cell lymphomas [80, 81].

Inactivation of the suppressor gene *p53* has been demonstrated in approximately 40% of AIDS–non-Hodgkin's lymphomas, including Burkitt's and Burkitt's-like lymphomas (Table 47.1). There is a lower frequency of *p53* mutations in large-cell immunoblastic lymphomas in individuals with and without AIDS [82]. *p53* mutations have been seen in both EBV-positive and EBV-negative tumors, suggesting no correlation between the tumorigenic mechanisms involving these two lesions [73, 83]. Conversely, the functional interdependence of accumulated genetic lesions in achieving tumorigenesis is exemplified by *p53* and c-*MYC* which frequently occur together [83]. In fact, elevated levels of mutant p53 have been shown to result from transcriptional activation of the *p53* promoter by an overexpressed c-MYC protein [84]. Deletion of two discrete regions on the long arm of chromosome 6, representing the sites of two putative tumor suppressor genes, is common in B-cell non-Hodgkin's lymphomas and constitutes a poor prognostic indicator [83]. Such 6q deletions are seen in 25% of AIDS–non-Hodgkin's lymphomas, suggesting a role for these putative genes in AIDS-related lymphomagenesis. Other commonly altered tumor suppressor genes have been examined in AIDS–non-Hodgkin's lymphomas. No mutations have been demonstrated for *RB1*, and the potential involvement of *NF*-1, *APC*, *DCC*, or *WT*-1 seems unlikely based on the lack of cytogenetic aberrations at the corresponding chromosomal regions in AIDS–non-Hodgkin's lymphoma [83].

Contribution of Viruses to AIDS-Associated Lymphomas

HIV and co-infecting viruses are believed to contribute to malignant transformation in the setting of AIDS. Over 90% of HIV-infected individuals are also infected with EBV. In most studies, Burkitt's lymphomas show inconsistent association with EBV (30% in systemic AIDS–Burkitt's lymphoma). Between 70 and 80% of AIDS–diffuse large-cell lymphoma variants (i.e., diffuse large B-cell and immunoblastic subtypes), on the other hand, are EBV positive. Interestingly, results have also shown that systemic AIDS–non-Hodgkin's lymphomas in which the tumor

clone is infected with EBV have a significantly increased risk of CNS involvement at some point during their clinical course, regardless of the molecular features of the EBV strain [85]. Furthermore, detection of EBV DNA in the CSF of patients with systemic AIDS–non-Hodgkin's lymphomas is highly predictive of a diagnosis of localized CNS involvement. A particularly strong association exists between EBV infection and Hodgkin's lymphoma and Ki-1+ anaplastic large-cell lymphomas in HIV patients as well [86].

EBV DNA is detected more frequently in monoclonal than polyclonal AIDS–non-Hodgkin's lymphomas in contrast to the polyclonal lymphomas that characterize other immunodeficient states [87]. The EBV genomes within the lymphoma clones are also clonal, indicating that the B-cell progenitor of each lymphoma was already infected with EBV before expansion. In vitro, HIV has been shown to transform EBV-immortalized B lymphocytes and to upregulate the expression of EBV and c-*MYC* in the transformed cells. Such HIV-transformed B cells are also able to give rise to lymphoproliferations resembling lymphomas when inoculated into nude mice [88].

In addition to EBV, other viruses have also been examined for their potential role in AIDS-related lymphomagenesis. Among these viruses, human T-cell leukemia virus (HTLV)-I and HTLV-II DNA sequences have been detected in lymphomatous tissues of AIDS patients. Herpesviruses other than EBV, namely, human herpesvirus type 6 (HHV-6, or HBLV), which is associated with lymphoproliferative disorders in both HIV-positive and HIV-negative individuals, and HHV-7, have been identified as well [89]. Cytomegalovirus (CMV), an opportunistic pathogen in virtually all individuals with AIDS, has also been shown to induce both T-cell proliferation and B-cell activation.

Given the high frequency of coinfection with HIV and other viruses, the possibility that different viruses interact synergistically to promote tumorigenesis should be explored. As an example, mitogenic stimulation of HIV-infected peripheral blood leukocytes by noninfectious HTLV-I virions results in the production of large amounts of HIV-1 [90]. Furthermore, many of the opportunistic viruses commonly seen in AIDS patients (including CMV, herpes simplex viruses, EBV, and HHV-6) encode transactivator proteins that upregulate transcription from the promoters of heterologous genes [91]. Thus, CMV encodes protein products that can transactivate the HIV long terminal repeat, or LTR [92]. A possible effect of this interaction is noted in cases of fulminant CMV infection, in which high amounts of HIV are also found. However, despite these potential contributing interactions among viruses prevalent in the setting of AIDS, the etiology of most of the AIDS-associated lymphomas is unknown.

Host Response

Host factors may also play a role in HIV patients' predisposition to lymphoma. Just as specific alleles of chemokines and their receptors are known to confer protection against or increased risk of HIV infection, an increased risk of HIV-associated non-Hodgkin's lymphoma, has been observed in association with a particular polymorphism of the stromal cell-derived factor 1 (SDF-1) chemokine gene [93, 94]. The risk is greater for homozygotes of this SDF1–3′A allele than for heterozygotes, with 19% of homozygous and 10% of heterozygous SDF1–3′A carriers developing lymphoma after a median follow-up of 12 years, in contrast to 5% of wild-type HIV-infected individuals.

Clinical Features of HIV-Associated Lymphomas

The most common HIV-associated lymphomas are DLBCL, Burkitt's lymphoma, and Hodgkin's lymphoma. Other subtypes, including primary effusion lymphoma and plasmablastic lymphoma, are also seen but less common. Some authors have noted considerable pleomorphism with frequent overlap between some of these histologic categories, suggesting a morphological spectrum of lymphomatous proliferations [95, 96]. Among the morphologic complexities noted within these larger categories are classical Burkitt's lymphoma (30%) and atypical Burkitt's-like lymphoma (among the small non-cleaved cell lymphomas), as well as lymphomas with features intermediate between Burkitt's lymphoma with plasmablastic differentiation and immunoblastic lymphoma [95]. Although more rare, other histologic types of lymphoma have also been noted in conjunction with HIV, including T-cell lymphomas, T-cell lymphoblastic leukemia, adult T-cell leukemia/lymphoma, peripheral T-cell lymphoma, and Ki-1+ anaplastic large-cell lymphoma.

In contrast to non-Hodgkin's lymphomas in immunocompetent individuals, a very high proportion of AIDS-associated non-Hodgkin's lymphomas present with extranodal disease, with or without nodal involvement. The most frequently involved sites are the CNS (20–40%), bone marrow (25%), and gastrointestinal tract (25%) [97]. Other affected sites include the skin, salivary glands, oropharynx, heart, lungs, liver, rectum, muscles, bones, orbit, mandible, and even the peripheral blood.

Approximately 31% of the AIDS-related non-Hodgkin's lymphomas are systemic. They usually present at advanced stages with up to 95% of cases being in stage III or IV [98]. Those few cases that are early stage are frequently extranodal (Stage IE and IIE disease) [98].

Diffuse Large B-Cell Lymphoma in HIV Patients

DLBCL, which accounts for over 70% of lymphoma cases, remains the main type of cancer that develops in patients with HIV, although the incidence has declined since the initiation of HAART [99]. DLBCLs are variable in their histology in patients with HIV. The centroblastic variant consists of diffuse sheets of large lymphoid cells with round or oval nuclei and prominent nucleoli. They are often positive for CD10 and CD20 and express Bcl-6, suggesting germinal center origin [100]. Alternatively, the immunoblastic variant refers to lymphomas with more than 90% immunoblasts with plasmacytoid features. CNS lymphoma typically falls into this category. These cells have plasma-cell surface markers, such as CD138, while still retaining features of mature B cells [100]. These tumors classically have frequent mitoses and high proliferation rates. The prognostic implications of these subtypes remain unclear.

Burkitt's Lymphoma in HIV Patients

Burkitt's lymphoma accounts for approximately 14% of AIDS-related NHL and is more commonly seen in patients with HIV than other types of immunodeficiency [101]. HIV-associated Burkitt's lymphoma cells typically express CD10, CD20 and have a proliferation rate close to 100%. Morphologically, Burkitt's lymphoma can fall into three categories. The classic type, which accounts for approximately 30% of patients, appears similar to those in non-HIV-infected patients [100]. Burkitt's lymphoma with plasmacytoid features, however, demonstrates pleomorphism in nuclear size and shape, abundant eccentric cytoplasm, and nuclei with a centrally located prominent nucleolus. Lastly, in atypical Burkitt's there is greater pleomorphism with fewer, but more prominent nucleoli [100]. This histologic appearance is typically only seen in patients with HIV [3].

Primary Central Nervous System Lymphoma

The incidence of primary CNS lymphoma of the brain, a rare tumor that accounts for less than 2% of all non-Hodgkin's lymphomas and 1% of all brain tumors, increased threefold from 2.7 to 7.5 cases per 10 million population between 1973 and 1984 [102]. Part of this increase may be attributed to the unusually high proportion, between 19 and 25%, of AIDS–non-Hodgkin's lymphomas that present as primary brain lymphoma (Table 47.1), with a frequency 1000 times greater than that in the general population [97]. Primary CNS lymphoma is the second most common AIDS-associated cerebral mass lesion, after toxoplasmosis. Sites of AIDS-associated CNS lymphomas include the cerebrum, cerebellum, basal ganglia, and brain stem [103]. Furthermore, the association that is generally seen between AIDS–non-Hodgkin's lymphomas and low CD4 counts is even more pronounced in primary CNS lymphomas, which tend to occur in patients with CD4 counts below 50 cells/mm^3 [104]. In contrast to primary brain lymphomas in the immunocompetent host, those occurring in conjunction with AIDS do not have a characteristic radiographic appearance. They can occur in the cortex or deep periventricular structures. Although 50–75% of cases are multifocal on computed tomography (CT) or magnetic resonance imaging (MRI) scan, at autopsy virtually all are multifocal. Twenty to 60% of AIDS-related primary CNS lesions show ring enhancement accompanied by surrounding edema, making them difficult to distinguish from toxoplasmosis and necessitating brain biopsy for accurate diagnosis [105]. Accompanying spinal fluid analysis may be normal or may show a mild pleocytosis that is primarily mononuclear, as well as elevated protein and depressed glucose [105]. It is notable that there has been a clear decrease in the incidence of AIDS-related PCNSLs since the advent of HAART [106].

Pathological evaluation usually shows a high-grade B-cell lymphoma. Although some studies note only diffuse large-cell histologies, especially large-cell immunoblastic lymphoma, other reports demonstrate small non-cleaved cell lymphomas among the primary CNS lesions. Leptomeningeal involvement has also been reported with high frequency. In contrast to the systemic AIDS–non-Hodgkin's lymphomas which show EBV infection in only 30–60% of cases, in situ hybridization has demonstrated EBV expression in up to 100% of primary CNS lymphomas in HIV patients [103]. The latter finding also differs from that in immunocompetent patients, in whom primary CNS lymphomas rarely contain EBV. Finally, primary localized extranodal lymphomas, like primary CNS lymphomas, tend to be immunoblastic, whereas both large-cell immunoblastic lymphomas and Burkitt's lymphomas have been noted in the meninges [107].

Hodgkin's Lymphoma in HIV-Infected Individuals

Although individual cases of Hodgkin's lymphoma in AIDS patients have been reported since 1984, Hodgkin's lymphoma has not been classified as an AIDS-defining illness to date. This omission was attributable to the failure of some studies to demonstrate an increased incidence of Hodgkin's lymphoma in HIV-infected patients. However, there now seems to be a clearer association with HIV, with at least a tenfold increased risk as compared to the general public [108]. This increased risk may even be greater since the implementation of HAART, as there is an increased incidence of Hodgkin's lymphoma in the post-HAART era.

The most prevalent histological subtype of Hodgkin's lymphoma reported among HIV-infected individuals is mixed cellularity. The nodular sclerosis type constitutes the second most common subtype (30%), though its frequency is decreased in comparison with non-HIV-infected patients with Hodgkin's lymphoma [109]. The proportions of both mixed cellularity and lymphocyte-depleted subtypes among HIV-infected individuals with Hodgkin's lymphoma (40–60% and 20%, respectively) are elevated relative to that in the non-HIV Hodgkin's disease population [108, 109]. Interestingly, in the post HAART era, there has also been a shift toward increased incidence of the nodular sclerosis subtype, which is seen more commonly with higher CD4 counts [110].

HIV-infected individuals with Hodgkin's lymphoma present with advanced (i.e., Stage III or IV) disease in as high as 80–90% of cases, often accompanied by B symptoms. Extranodal disease occurs in two-thirds of patients, with bone marrow involvement in up to 50%, in contrast to the 3.5% observed in patients with Hodgkin's lymphoma not infected with HIV [111, 112]. Other reported sites of involvement include lung, liver, bone, paraspinal masses, rectum, and CNS, as well as other unusual sites such as the tongue and skin. A relative paucity of mediastinal disease has been noted in HIV-infected patients—22 versus 50% in the absence of HIV infection—and noncontiguous spread may occur in the setting of HIV [113]. In contrast to patients with seronegative disease, patients with HIV Hodgkin's lymphoma are nearly always associated with EBV [108].

Body Cavity Lymphoma/Primary Effusion Lymphoma

An unusual clinical entity, *body cavity-based lymphoma* associated with lymphomatous effusions (pleural, pericardial, or peritoneal) in AIDS patients was originally described in 1989 and was subsequently designated *primary effusion lymphoma* [114]. Knowles et al. have noted such lymphomatous effusions in the absence of a contiguous tumor mass in MSM patients usually, but not always, in association with HIV infection [115]. The lymphomatous effusions generally constituted the initial presentation of a lymphoma, which in most cases remained localized to the body cavity of origin. Examination of the malignant cells revealed features of both large-cell immunoblastic lymphoma and anaplastic large-cell lymphoma. Despite the absence of B-cell-associated antigens, clonal Ig gene rearrangements placed these lymphomas in the B-cell lineage. A potential etiologic agent for these body cavity-based lymphomas was identified when a DNA sequence homologous to a 233-base pair region of Kaposi's sarcoma-associated herpesvirus-like virus (KSHV) was observed in all AIDS-related body cavity-based lymphomas associated with lymphomatous effusions but in none of the other tested AIDS- or non-AIDS-related lymphomas [116]. Subsequently, KSHV sequences have been identified in body cavity-based lymphomas in patients not infected with HIV, including patients with Kaposi's sarcoma, post-cardiac transplant, and even in immunocompetent individuals. The putative agent, KSHV, named for its original association with Kaposi's sarcoma lesions in AIDS patients, is now classified as human herpesvirus-8 (HHV-8) based on homology of KSHV sequences to members of the Gammaherpesvirinae subfamily of herpesvirus [117]. Similar to EBV, KSHV/HHV-8 exists in the latently infected cells of primary effusion lymphoma as multiple copies of a circularized DNA episome. A KSHV/HHV-8-encoded latency-associated nuclear antigen (LANA) appears to be necessary and sufficient for the persistence of these episomes, tethering KSHV/HHV-8 DNA to chromosomes during mitosis to ensure their efficient segregation to progeny cells [118]. In addition to KSHV/HHV-8, EBV sequences have been identified in well over 70% of body cavity-based lymphomas examined [119]. The combined presence of KSHV/HHV-8 and EBV in this particular presentation of lymphoma supports a synergistic effect in which both agents act as carcinogenic insults contributing to the multistep progression toward malignant lymphoma.

Treatment of AIDS-Associated Lymphomas

Before use of HAART, patients with HIV-associated lymphomas had poor outcomes, given chemotherapy failures and high rates of infection. Prognosis in these patients was determined primarily by their performance status and severity of HIV rather than the type of lymphoma. While a variety of dose and risk adjusted treatments were studied, median survivals remained at approximately 5–6 months [100]. Since the advent of HAART, patients have had dramatic improvements in outcome. Given improvements in immune function, patients are now able to tolerate optimal chemotherapy regimens with outcomes similar to those in non-HIV-infected patients.

The decision about timing of HAART treatment remains incompletely established, primarily because of issues related to potential drug interactions. Components of the HAART regimen, particularly the non-nucleoside reverse transcriptase inhibitors (NNRTIs) and the protease inhibitors (PIs), are extensively metabolized by the cytochrome P-450 (CYP450) enzyme system. These drugs may also be inhibitors or inducers of CYP450. Since many chemotherapeutic agents are also metabolized by the CYP system, concomitant HAART use presents theoretical disadvantages. For example, concomitant HAART and chemotherapy might result in either drug accumulation and possible toxicity, or decreased

efficacy of one or both groups of agents. Nonetheless, there is now considerable experience of concomitant use, even in dose-intense regimens, and most guidelines recommend concurrent use.

There has also been controversy regarding the role of rituximab in addition to chemotherapy in patients with HIV-associated NHL. While there is a clear benefit in patients with non-HIV-related disease, the increased risk of infection in patients with HIV has raised concern about its universal use. One randomized controlled trial did not demonstrate a benefit in survival with the addition of rituximab to cyclophosphamide, doxorubicin, vincristine, and prednisone (CHOP) for patients with NHL, presumably given an increase in infection related mortality [120]. Subsequent studies, however, have demonstrated safety and efficacy with rituximab use with CHOP and dose escalated cyclophosphamide with etoposide, vincristine, doxorubicin, prednisone (da-EPOCH) in this population [121]. Based on these studies, rituximab use is recommended, though should be used with caution in the setting of CD4 counts <50/uL.

Controlled studies investigating the role of CNS prophylaxis in HIV-positive patients with lymphoma are also lacking. In HIV-negative patients, there is an increased risk of CNS relapse in patients have an elevated LDH, ECOG performance status greater than 1, and extranodal involvement. It is therefore reasonable to provide CNS prophylaxis for patients in HIV-positive patients with these features as well. Options for prophylaxis include high-dose methotrexate and liposomal extended release cytarabine [122].

Incorporation of granulocyte/macrophage colony-stimulating factor (GM-CSF, sargramostim) or granulocyte colony-stimulating factor (G-CSF, filgrastim) into the chemotherapeutic regimens has permitted the use of standard doses with the maintenance of higher mean nadirs of absolute neutrophil count, shorter mean durations of neutropenia, and fewer episodes of neutropenic fever. However, no statistical difference in survival time has been demonstrated as a consequence of CSF use [123]. Furthermore, evidence from elevated mean p24 levels suggests that GM-CSF stimulates HIV replication [123]. However, the elevation is transient and is not consistently observed.

For patients with relapsed or refractory AIDS–NHL, standard treatment remains to be established. Thus, patients are often treated in a manner similar to that of HIV-negative patients, such as with isofamide, cisplatin, etoposide (ICE), or rituximab-ICE or with etoposide, solumedrol, high-dose cytosine arabinoside, and plantinum (ESHAP). Patients responsive to second-line chemotherapy have been successfully treated with high-dose chemotherapy and autologous stem cell transplant. In one report, 84% of such patients (n = 19) were in complete remission at a median follow-up of 27 months (range 6–57 months) [124]. In a multi-institutional study conducted by the AMC, of 20 patients, 10 (50%) were event free and 13 remained alive at a median follow-up time of 23 weeks [125].

While outcomes for patients with HIV Hodgkin's lymphoma have improved dramatically since the advent of HAART, standard therapy in this population has not been defined. Typically, a standard chemotherapy regimen for patients with Hodgkin's lymphoma in non-HIV patients such as doxorubicin, bleomycin, vinblastine, and dacarbazine (ABVD) is used. Alternatively, a stage and risk-adapted approach, which has been described by German investigators, also seems to be efficacious in this population [126]. With this strategy, patients with early favorable disease are treated with two to four cycles of ABVD followed by intensive field radiation. Patients with early unfavorable disease receive four cycles of bleomycin, etoposide, doxorubicin, cyclophosphamide, vincristine, procarbazine, and prednisone (BEACOPP) or ABVD with radiation. Lastly, patients with advanced stage disease receive six to eight cycles of BEACOPP. Given the toxicity of the BEACOPP regimen, ABVD is typically preferred in patients with advanced HIV.

There are limited data to suggest an optimal treatment regimen in HIV-associated Burkitt's lymphoma. Dose intensive regimens used in the treatment of acute lymphoblastic leukemia (ALL) have been applied to HIV-associated Burkitt's lymphoma with outcomes similar to patients without HIV. Other dose intensive regimens such as Hyper-CVAD/HD-MTX and CODOX-M-IVAC regimens that are used in seronegative patients have also been used. Given significant toxicities with this regimen, dose adjusted R-EPOCH may be preferred for some patients [121]. Central nervous system prophylaxis is also typically included in this population.

Other Viruses

In addition to EBV and HIV, HTLV-I, HHV-8, and HCV are also strongly implicated as lymphomagenesis. HTLV-I is a single-stranded RNA virus that integrates randomly into the host cell genome, with one copy of this provirus being detectable per cell. The HTLV-I genome codes for Tax, a viral transcriptional transactivator that upregulates viral and cellular gene products that may contribute to carcinogenesis [127, 128]. In HTLV-I-infected cells, for example, Tax activates *NF-κB* and $BCL\text{-}X_L$, which leads to the inhibition of apoptosis and ultimately results in an increase in cytokine production and cell proliferation.

The majority of HTLV-1-infected individuals remain asymptomatic, though approximately 5% ultimately develop the rapidly progressive T-cell leukemia/lymphoma, ATLL, characterized by uncontrolled clonal proliferation of mature transformed CD25 + CD4+ T cells and a very poor prognosis [129]. Four subtypes of ATLL are seen: acute, chronic,

smoldering, and lymphoma type [130]. All subtypes have viral DNA clonally integrated into the cellular DNA of the neoplastic cells. The acute form of ATLL is an aggressive T-cell lymphoma associated with a high white blood cell count, hypercalcemia, hepatosplenomegaly, and lytic bone lesions. The presence in the peripheral blood of large flower-like cells with lobulated nuclei is pathognomonic for ATLL. The chronic form resembles the acute form in some ways (e.g., hypercalcemia) but lacks the characteristic peripheral blood involvement of ATLL. Smoldering ATLL may resemble mycosis fungoides/Sezary syndrome in that cutaneous involvement occurs in the form of erythema or infiltrative plaques or tumors. Both the acute and the lymphoma-type ATLL have a poor prognosis, with mean survival being only 6 months. They are poorly responsive to conventional chemotherapy, encouraging the use of experimental approaches such as aggressive infusional chemotherapy which has shown some promise, yielding up to 93% overall objective response rate with a median survival of 17.4 months [131]. HTLV-I is endemic in areas such as southern Japan, the Caribbean Islands, and some areas of Brazil, where ATLL accounts for over half of adult lymphoid malignancies. In contrast to HTLV-I, HTLV-II has not been strongly correlated with any disease.

HHV-8/KSHV, in addition to showing an association with body cavity-based lymphomas in the setting of HIV (see earlier discussion), has been observed in the plasma cell variant of multicentric Castleman's disease and in angioimmunoblastic lymphadenopathy (AILD).

As a final example, the prevalence of chronic HCV infection has been shown to be higher in patients with B-cell non-Hodgkin's lymphoma than in controls, suggesting a potential role for HCV in B-cell lymphomagenesis [132]. HCV is a single-stranded, RNA virus that infects liver, and in a subset of approximately 20%, causes irreversible liver disease in the form of either cirrhosis or hepatocellular carcinoma. HCV is implicated in extrahepatic disease as well. For example, it is the primary etiologic agent of type II mixed cryoglobulinemia, which is associated with an underlying B-cell proliferation in 2–11% of cases. Although an association between HCV infection and non-Hodgkin's lymphoma has not been observed by all investigators, in a retrospective cohort study conducted within the Department of Veterans Affairs including 146,394 individuals infected with HCV and 572,293 not infected, there was a 20–30% increased risk of NHL and an almost threefold increased risk of Waldenstrom's macroglobulinemia among those infected with HCV [133]. Furthermore, a meta-analysis which included 15 case–control and three cohort studies revealed a pooled risk ratio (relative risk) of 2.0 (95% CI, 1.8–2.2) for the cohort studies and 2.5 (95% CI, 2.1–3.1) for the case–control studies for the development of B-cell NHL in HCV-infected patients [134]. Although most studies have concentrated on the association of HCV with B-cell lymphomas, a meta-analysis of nine published reports also demonstrated a significant association with T-cell lymphomas [135].

Mucosa-Associated Lymphoid Tissue (MALT) Lymphoma

Of the many lymphomas associated with infectious agents, those that arise in mucosa-associated lymphoid tissue (MALT) provide an excellent model of antigen-driven malignancy in which environmental factors and host immune response are demonstrably involved in the pathogenesis of lymphoma. MALT lymphomas are extranodal marginal zone B-cell lymphomas most commonly arising in the stomach but also occurring in the lung, ocular adnexa, skin, salivary glands, thyroid tissue, and breast. MALT lymphomas occur in the context of chronic inflammation related to infectious disease, including *Helicobacter pylori*, *Borrelia burgdorferi*, and *Chlamydophila psittaci*, or autoimmune disorders including Sjögren's syndrome or Hashimoto thyroiditis.

Helicobacter pylori

For many years, the belief was held that microorganisms could not inhabit the stomach and that gastric juice was sterile. More recently, however, not only was a high prevalence of *H. pylori* shown in the stomachs of the population, but its presence has also been associated with disease. *H. pylori* eradication has become a primary component of the therapy of peptic ulcer disease and non-ulcer dyspepsia, in which it has been shown to play an etiologic role. In addition, infection with the organism appears to be a risk factor for gastric carcinoma and lymphoma, raising the prospect that these cancers could be prevented by treating the infection.

The stomach wall normally contains no lymphocytes. However, in persons infected with *H. pylori*, a lymphoid cell infiltrate, *MALT*, may appear and eventually develop into a lymphoma. MALT lymphoma, the most common type of marginal zone lymphoma, is mucosal based, showing a tropism for the epithelium of the affected extranodal parenchyma [136]. The key histologic feature is the presence of lymphoepithelial lesions, with invasion and partial destruction of the gastric glands or crypts by aggregates of centrocyte-like cells [137]. Although low-grade MALT lymphomas are generally believed to be localized (Stage I_E or II_E) at the time of diagnosis, some studies have demonstrated dissemination in up to a third of cases [136, 138].

The mechanism of the lymphomagenesis is incompletely understood, but the infecting organism, *H. pylori*, is believed to provide an antigenic stimulus to neoplastic B cells in the

lymphoid infiltrates via *H. pylori*-specific T-cell proliferation [139]. In addition, host T-cell regulatory gene polymorphisms could influence T-cell response in gastric MALT lymphoma [140]. The subsequent development of genetic alterations also contributes to carcinogenesis. The loss of chromosome 3 has been suggested as playing a role in the transition from benign MALT to low-grade MALT lymphoma [141]. Similarly, trisomy 3 and, less frequently, trisomy 18 have been observed in most MALT lymphomas.

Three major translocations specific of MALT lymphomas are reported which share a common pathogenic mechanism: activation of the NF-kB signaling pathway. The t(11;18)(q21;q21) is the most common (20–30%) and is associated with *H-pylori* eradication unresponsive gastric MALT lymphoma [142].

Another genetic abnormality encountered in 1–2% of MALT lymphomas is the t(1;14) translocation. The t(1;14) translocation results in truncation of *Bcl*-10, leading to activation of *NF*-κ*B* and absence of apoptosis [143]. Similar to the t(1;18), the t(1;140) MALT lymphoma is usually advanced and unresponsive [144].

The t(14:18)(q32q21)/(IGH-MALT1) translocation occurs in 10–20% of MALT lymphomas, mostly arising in the skin (14%) and ocular adnexa (13%) [145]. This translocation is typically associated with additional chromosomal aberrations including trisomy 18 and with more aggressive clinical behavior.

Although c-*MYC* rearrangements are not generally seen in MALT lymphomas, mutations in exon 1 and the intron 1 regulatory region were observed in 17% of cases examined, implicating this oncogene in MALT lymphomagenesis [138].

Moving along the carcinogenic spectrum, the relationship of low-grade to high-grade MALT lymphomas is complex. One possibility is that low-grade MALT lymphomas progress to diffuse large-cell lymphomas. Such a relationship is supported by epidemiological data linking the much more common high-grade gastric diffuse large-cell lymphoma to *H. pylori* infection. In fact, most gastric MALT lymphoma patients (98.5%) are seropositive for *H. pylori*, although only 58–78% of resected specimens actually contain the *H. pylori* bacteria (63% for low-grade vs. 38% for high-grade lymphomas) [146]. Histologic distinction of grade among MALT lymphomas, however, has posed problems, with many presentations occurring as mixtures of low- and high-grade tumors. Therefore, attempts to determine the existence of a transition from low- to high-grade have relied on documenting changes at the molecular level.

A sense of transition from low- to high-grade MALT lymphoma emerges from comparisons of *p53* mutations (seen in 6%, 12%, and 31% of low-, mixed-, and high-grade tumors, respectively) and *bcl-2* overexpression (seen in 93, 88, and 44% of low-, mixed-, and high-grade tumors, respectively), which are inversely correlated with each other [147]. These data point to a possible association of *p53* mutations with transformation to high-grade lymphoma. Another genetic alteration that appears to correlate with progression to more aggressive histology is mutation of the *BCL*10 gene [145].

Low-grade gastric MALT lymphomas have been successfully treated by eradication of *H. pylori* with antibiotic therapy. Complete regression of low-grade MALT lymphomas occurs in 50–80% of the treated cases. More recently, patients with early stage high-grade gastrointestinal lymphomas experienced complete remission after eradication of *H. pylori* as well [148]. When antibiotic therapy is unsuccessful or is inappropriate, surgical resection or radiation has been administered, since low-grade MALT lymphoma is usually confined to the site of origin. Chemotherapy has also been administered with good results. To date, there is no evidence from controlled trials defining optimal therapy, and none of the modalities has been proven superior to the others in terms of prolonging survival.

Chlamydophila psittaci

The occurrence of ocular adnexal MALT lymphoma (OAML), a marginal zone B-cell lymphoma, has increased in recent years, with its highest prevalence among older women [149, 150]. OAML display typical histopathology and immunophenotype profile of MALT lymphomas with clonal IGHV gene rearrangement in 55% of cases [151]. Although MALT-related chromosomal translocations are uncommon in OAML (<10% of cases) [642], trisomy 3 and 18 occur frequently [152].

Chlamydiae are ubiquitous, obligate intracellular bacteria responsible for a wide range of human diseases, including chronic conjunctivitis. *Chlamydophila psittaci* (CP) has been detected by PCR techniques in 80% of Italian patients with OAML [153]. Retrospective reviews from Korea and Austria reveal similar, but not quite as high, infection rates among OAML patients [154, 155]. Other eye pathogens, such as *C. trachomatis* and herpes simplex viruses 1 and 2 have not been implicated in OAML; however, *C. pneumonia* has been detected in a few cases.

In addition to OAML, *Chlamydophila psittaci* has been detected in a significant portion of DLBCL arising in the skin and Waldeyer's ring, although it does not appear to be involved in the pathogenesis of lymphomas in the gastrointestinal tract or in pulmonary MALT lymphoma.

Borrelia burgdorferi

An association between primary cutaneous B-cell lymphoma (CBCL) and infection with the spirochete *Borrelia burgdorferi*,

the infectious cause of Lyme disease, has been observed in a number of cases. A pathogenetic role for the organism was suggested in part by the clinical observation of B-cell lymphomas arising on skin affected by acrodermatitis chronica atrophicans, a skin lesion known to be caused by *B. burgdorferi*. Raised titers of antibodies to *B. burgdorferi* in the sera of patients with CBCL offered additional evidence supporting such an association [156]. Monoclonality of the lymphomatous skin infiltrate has been demonstrated, and specific DNA sequences of *B. burgdorferi* have been identified in the cutaneous lesions in 18% of cases. The lymphomas are generally low-grade, extracutaneous involvement is rare, and progression is slow, with tumors remaining confined to the skin for as long as 7–15 years. By analogy to *H. pylor*i-associated MALT lymphomas of the stomach, a proportion of *B. burgdorferi*-associated lymphomas have been cured by appropriate antibiotic treatment, with concomitant eradication of the spirochete [156, 157]. Reports have also suggested an association between *Borrelia burgdorferi* and some cases of mantle cell lymphoma, mycosis fungoides, and possibly immunoproliferative small intestinal disease (IPSID).

Lymphomas in the Setting of Transplantation

Lymphomas in the Setting of Solid Organ Transplantation

Epidemiology/Demographics of Lymphomas Associated with Organ Transplants

Organ transplantation and the immunosuppressive therapy that is often essential to prevent rejection of transplanted organs, have been found to contribute to the development of non-Hodgkin's lymphomas. This is especially true of solid organ transplants where patients have a twofold risk of developing cancer compared to the general public [158]. Excluding non-melanoma skin cancers and in situ carcinomas of the cervix, the most frequently encountered malignancy in the transplant setting is lymphoma, which comprises 14% of posttransplant cancers [158]. Up to 25% of transplant patients develop post-solid organ transplantation lymphoproliferative disorders (PTLD), depending on the organ transplanted [159–161]. PTLDs are clinically and pathologically a heterogeneous group of lymphoid proliferations, most of which are of B-cell origin and are associated with EBV [161]. Despite the heterogeneity, PTLD remains a serious complication of transplantation with a 5-year overall survival ranging from 40 to 70% [162, 163].

The average time of occurrence of all cancers following transplant is 61 months. Lymphomas tend to appear earlier, averaging about 36–40 months [163]. EBV-negative PTLD, however, presents later, with occurrences more than 1 year following transplant [163].

Additional demographics point to a higher incidence of posttransplant lymphomas in North America than in Europe among both kidney and heart transplant patients [164]. In the United States, the risk of developing non-Hodgkin's lymphoma is slightly lower among African–American than white patients [164]. Several of these epidemiologic correlates probably reflect two key features of transplantation: the type of transplant and the nature of the immunosuppressive therapy.

Influence of Type of Transplant on Lymphoma Incidence

Risk of PTLD has been linked to pretransplant EBV mismatch, the immune suppression agents used and the type of organ transplanted. The incidence of PTLD is highest following intestinal or multi-organ transplants (up to 20%), followed by lung (2–9%), heart (2–6%), liver (1–2%), and renal transplants (1–3%) [165–167]. It is thought that these differences arise from variation in histocompatibility as well as the amount of lymphoid tissue present in the transplanted organ. For example, the small intestine, which has high rate of PTLD, has significantly more lymphoid tissue and subsequently donor lymphocytes [168]. Interestingly, analysis of sex chromatin, HLA specificities, and DNA restriction fragment length polymorphisms indicates that most posttransplant lymphomas following solid organ transplantation derive from recipient rather than donor lymphocytes.

Influence of Immunosuppressive Therapy on Lymphoma Incidence

A number of immunosuppressive agents, including azathioprine, cyclophosphamide, tacrolimus, and cyclosporine, have been shown to promote tumorigenesis in the transplant setting [161]. It is theorized that the reduction in host cytotoxic T-cell response decreases control of EBV infection of the transplanted graft, thus predisposing to PTLD. While it is difficult to dissect out the tumorigenic effect of a single component, qualitative differences have been demonstrated among these agents with respect to their carcinogenic, and specifically lymphomagenic, potential. In kidney transplant patients, for example, immunosuppression with cyclosporine did not increase risk of PTLD compared to azathioprine and steroids, but treatment with tacrolimus led to an approximately twofold risk [164]. Maintenance tacrolimus has also been associated with increased risk of disease as compared to cyclosporine [169].

The degree of immunosuppression also clearly influences risk of developing PTLD. Thus, the higher incidence of LPD seen in early trials with cyclosporine relative to established immunosuppressive therapy was subsequently traced to the higher cyclosporine doses used at the time [170]. Higher cumulative doses of prophylactic anti-CD3 (more than 75 mg) in cardiac transplant recipients correlate with a higher incidence of lymphomas, 35.7%, compared with 6.2% for

doses less than 75 mg [166, 171]. Anti-CD3 doses higher than 75 mg also correlate with a shorter time interval between transplant and development of lymphoma, averaging only 1–2 months. Furthermore, the variable incidence of malignancy, and specifically lymphoma, in different subgroups of transplant recipients may at least in part be attributable to the intentional implementation of more intensive immunosuppressive regimens in certain settings. Termination of immunosuppressive therapy may also be a reasonable option for management of severe rejection of a kidney transplant, given the non-life-threatening nature of the transplant rejection. However, no such option is available when a comparable threat of rejection occurs in the setting of heart, liver, or lung transplant, necessitating intensification of the immunosuppressive regimen in order to save the patient's life.

In addition to higher doses of individual agents, the use of combinations of immunosuppressive agents to minimize individual toxicities may carry its own risk by undermining different points in the immune system [172]. Doses of cyclosporine used in triple therapy with prednisone and azathioprine, for example, have been lowered in an effort to reduce nephrotoxicity. Despite these reduced cyclosporine doses, the incidence of lymphoma is increased with triple therapy.

Clinical Features and Histopathology

PTLD displays a spectrum of lesions ranging from polymorphic diffuse B-cell hyperplasia to intermediate polymorphic diffuse B-cell lymphoma to true monomorphic large-cell immunoblastic lymphoma [173–175]. The polymorphic presentations, which contain small and large cells of both follicular center and medullary type, have been interpreted as benign reactive hyperplastic or neoplastic lymphomas depending on whether cell marker studies reveal polyclonal or monoclonal B-cell proliferations [176]. Evolution from a polyclonal polymorphic lymphoproliferation to a true monoclonal monomorphic large-cell immunoblastic lymphoma has been demonstrated with individual lymphomatous sites displaying distinct monoclonal derivations. Similarly, PTLD lesions have been described in which polyclonality is suggested by immunophenotyping but that contain either Ig gene rearrangements or clonal cytogenetic abnormalities [177]. This suggests that independent activation of multiple cells is occurring as an early step in tumorigenesis, with occasional early malignant transformation detectable at the DNA or cytogenetic level. Among the true monomorphic monoclonal lymphomas, several histopathologic types have been identified, including large-cell immunoblastic lymphoma, DLBCL, diffuse mixed lymphoma, and small noncleaved Burkitt's and non-Burkitt's lymphoma.

Initially based on morphologic categories, the classification system for PTLDs was subsequently modified to incorporate correlative genetic and molecular analysis [178]. Knowles and colleagues incorporated a series of attributes that jointly define three categories of PTLDs (Table 47.2): (1) plasmacytic hyperplasias, which are polyclonal, associated with multiple EBV infections, and lack oncogene or

Table 47.2 Histopathologic categories of posttransplantation lymphoproliferative disorders[a,b]

Category of lymphoproliferation	Anatomic site(s) at presentation	Clonality by Ig gene	EBV events	Oncogene, tumor suppressor gene alterations	Clinical intervention and outcome
Plasmacytic hyperplasia	Oropharynx, lymph nodes	Most polyclonal	Most with multiple EBV infection events, or only a subset of cells infected by a single form of EBV, or EBV negative	Absent	All responded to reduced immune suppression + surgical resection
Polymorphic B-cell hyperplasia and polymorphic B-cell lymphoma	Lymph nodes, various extranodal sites	Most monoclonal	Most with single form of EBV	Absent	Respond to reduced immunosuppression + surgical resection; or to medical therapy; or no response
Immunoblastic lymphoma, multiple myeloma	Widely disseminated disease	Monoclonal	Single form of EBV	Alteration of one or more oncogenes or tumor suppressor genes: N-*ras*, *c-myc*, *p53*	Medical resolution; or no response—Most

[a]Data from Knowles DM, Cesarman E, Chadburn A et al. Correlative morphologic and molecular genetic analysis demonstrates three distinct categories of posttransplantation lymphoproliferative disorders. *Blood*. 1995;85(2):552–65; and from Chadburn A, Chen JM, Hsu DT. The morphological and molecular genetic categories of posttransplantation lymphoproliferative disorders are clinically relevant. *Cancer*. 1998;82:1978–1987

[b]Based on data from 1 lung, 5 kidney, and 26 heart transplant recipients

tumor suppressor gene alterations; (2) polymorphic B-cell hyperplasias and polymorphic B-cell lymphomas, which are generally monoclonal, manifest a single form of EBV, but still lack genetic changes in oncogenes or tumor suppressor genes; and (3) immunoblastic lymphomas or multiple myelomas, which are monoclonal, contain a single form of EBV, and exhibit alterations in one or more oncogenes or tumor suppressor genes [178]. Additionally, these tumor types tend to have cytogenetic karyotype abnormalities, including trisomy 9, trisomy 11, and rearrangements involving 8q24 [179]. An attempt to validate this system in terms of its clinical relevance has revealed that these categories correlate with clinical presentation, response to therapy, remission, and clinical outcome [180]. Importantly, all lesions categorized as plasmacytic hyperplasia regressed in response to reduction in immunosuppression alone or accompanied by surgery. Polymorphic PTLDs that did not regress with reduction in immunosuppression usually resolved with medical intervention, such as chemotherapy or radiation therapy. Lesions classified as malignant lymphoma/multiple myeloma did not respond in spite of aggressive clinical intervention. These histologic-clinical correlates provide a basis for choosing optimal treatment in the management of PTLDs.

Although about 85% of posttransplant lymphomas are B cell in origin, occasional T-cell lymphomas have been identified, some of which have been linked to EBV infection [181–183]. A rare CD8+ γδ T-cell lymphoma reported in the setting of renal transplant was found to be associated with human HHV-6 infection [184]. B- and T-cell lymphomas have also been observed within a single patient, and T-cell receptor and Ig heavy chain rearrangements have even been detected within the same lymphoid proliferation in a renal allograft, indicating a polyclonal origin to the PTLD [185, 186]. Unusual presentations such as that of a nasal natural killer cell lymphoma associated with EBV have also been reported post-renal transplant [187]. MALT-type lymphomas have been observed in the stomach and parotid gland following cardiac, liver, or kidney transplant [188]. Interestingly, all cases examined were negative for EBV, and the gastric lymphomas were all positive for *H. pylori*, resembling MALT lymphomas in immunocompetent patients rather than lymphomas that occur in association with immunosuppressed states. Hodgkin's disease has only been reported uncommonly following kidney, liver, and heart transplants [189]. Posttransplant leukemias occur but are uncommon, and most of them are granulocytic, particularly monocytic, in phenotype [190]. Other lymphoid-related proliferative diseases that are occasionally observed in the posttransplant setting include monoclonal gammopathies, multiple myeloma, and plasmacytomas [191].

As with lymphomas in other settings of immunosuppression, posttransplant lymphomas frequently present at advanced stages. Thus, in one study 50% were diagnosed as Stage III or IV, while 35% were Stage I [182]. The similarity to other immunodeficiency states extends to the frequency with which the posttransplant lymphomas occur at extranodal sites. Between 26 and 45% occur in the CNS: 14–66% of these are confined to the brain parenchyma with frequent multifocality and virtually no lymphomatous meningitis [161, 182].

The allograft has also been described as a site of involvement by lymphoma in 15–31% of cases, the lymphomatous infiltrate sometimes being mistaken on biopsy for graft rejection [192]. In fact, Opelz et al. noted a strong preferential location of lymphomas in the heart or lung after heart transplantation and in the kidney after renal transplantation [164]. On the other hand, Leblond et al. have suggested that allograft presentation may be less common in heart than in renal transplants [193]. Presentation in the allograft brings to mind the question of whether the tumor or a tumorigenic agent such as EBV (see discussion to come) was in fact transmitted from the donor, having been transplanted with the graft.

Pathogenesis: Mechanisms Underlying Lymphomagenesis in Association with Solid Organ Transplants

Molecular and Immunologic Basis for Posttransplant Lymphomagenesis

PTLD is believed to arise by a series of carcinogenic steps that roughly correspond to those described previously for HIV- and EBV-related lymphomas. Malignant progression is facilitated by a variety of mechanisms, some of which are unique to the setting of organ transplant. Thus, the underlying disease process that necessitates the transplant may predispose the patient to malignancy. As an example, a history of polycystic kidney disease seems to correlate with the development of posttransplant lymphoma [194]. By contrast, the duration of renal disease and length of time on dialysis, both potential contributors to overall immunosuppression, appear to play no role in lymphomagenesis, although they may influence the frequency of other malignancies [172, 195]. Among cardiac allograft recipients, PTLD has been noted primarily in individuals transplanted for idiopathic cardiomyopathy, rather than coronary artery disease [196, 197]. The reasons for this difference are unclear. Idiopathic cardiomyopathy is associated with a defect in mitogen-stimulated suppressor T-cell activity, whereas coronary artery disease is not; however, the relationship of this finding to subsequent lymphomagenesis remains obscure [198].

Initial B-cell proliferation results from exposure of host lymphoid tissue to a number of foreign antigenic stimuli, some of which are peculiar to the transplant setting. Chronic exposure to foreign histocompatibility antigens in the allograft may lead to hyperplasia [199, 200]. Blood transfusions, heterologous ATG or ALG, monoclonal antibodies like anti-CD3, and various infectious agents may elicit proliferative responses in lymphoid tissues. In particular, EBV, which is highly associated with the development of posttransplant lymphomas (see discussion to come), provides a potent antigenic stimulus to lymphoproliferation, as discussed previously. Patients with PTLD also often have decreased levels of interferon-α, a downregulator of IL-4, in turn allowing increased expression of IL-4, a potent stimulator of B-cell proliferation [201].

Within the expanded polyclonal B-cell population, genetic alterations occur that may lead to clonal expansion and malignant transformation. Although these changes have not been as extensively documented as in the AIDS–non-Hodgkin's lymphoma literature, clonal cytogenetic abnormalities suggestive of malignant progression have been identified in posttransplant lymphomas. For example, within polyclonal B-cell proliferations, cellular clones containing a 6q + long marker chromosome, trisomies 3 and 14, t(9;14) involving the IgH locus on chromosome 14, and t(2;19) involving the Ig-κ light chain locus on chromosome 2 have been identified [173, 202–204]. More recently, recurring copy number amplification in 9q24.1 have been seen in EBV-positive disease. Amplification of this locus, which includes programmed death ligand 1 and 2, may contribute to tumorigenesis by suppressing the anti-tumor immune response by T cells [205].

Finally, iatrogenic immune suppression further contributes to multistep tumorigenesis, by permitting the unchecked expansion of malignant clones. Each of the immunosuppressive agents used to prevent organ rejection targets discrete sites along the cascade of T-cell activation. For example, azathioprine plus prednisone abolishes natural killer cell-mediated cytotoxicity [206]. Glucocorticoids inhibit the expression of a number of cytokines, including IL-1, IL-3, IL-6, interferon-γ, and tumor necrosis factor-α, resulting in depression of T-cell proliferation and T-cell-mediated immunity. Cyclosporine blocks IL-2 gene activation, IL-2 receptor expression, and IL-2- and IL-6-mediated proliferation of mitogen-activated T cells [207]. Conversely, cyclosporine induces expression of IL-6, an activator of B-cell proliferation [207]. By thus modulating these two interacting arms of the immune system, cyclosporine may contribute to depressed T-cell surveillance of an expanding B-cell population in transplant recipients.

Role of EBV in Posttransplant Lymphomagenesis

A large volume of data supports the association of EBV infection with PTLD. First, EBV levels seem to increase in patients following transplantation. Renal transplant recipients, for example, exhibit higher levels of EBV oropharyngeal shedding than do healthy seropositive controls [208]. B cells containing EBV genomic DNA, which normally exist in the peripheral blood of seropositive individuals at concentrations of less than $1/10^7$ peripheral blood mononuclear cells (PBMCs), also increase in transplant recipients to more than $1/10^5$ PBMCs 1–3 months after transplant and remain high for about 3 months [209]. Certain studies suggest that the presence of increased levels of EBV DNA or RNA in lymphoid cells correlates with the subsequent development of PTLD, though the data has been conflicting [209–211]. Finally, EBNA-2 and EBNA-3C DNA sequences typifying EBV type 1, the more potently transforming strain (see earlier discussion), have been found in the majority of EBV-positive disease [212].

Transmission of EBV from a seropositive donor via the allograft leads to primary infection in virtually all initially seronegative transplant recipients. Swinnen et al. observed that among patients developing PTLD, 30% had negative tests for EBV before transplantation [171]. This observation, together with those from other studies involving a variety of transplanted organs, suggests that initial EBV seronegativity, with resulting primary EBV infection, is an important risk factor for PTLD [166]. In fact, the simultaneous development of PTLD in two seronegative recipients of kidneys from a single seropositive donor has been reported [213]. In addition to recipient seronegativity, donor EBV positivity has been shown to predict for seroconversion and developing PTLD [214]. The association of baseline seronegativity with conversion via primary infection as a risk factor for PTLD is especially pertinent to transplantation in the pediatric age group, since younger patients are more likely to be EBV seronegative. Data on pediatric transplant recipients bear this out, with symptomatic mononucleosis-like EBV infection and PTLD occurring more frequently in this age group than among adults [214, 215]. Because of the strong association of EBV transmission and primary EBV infection with the development of PTLD, some investigators believe that seronegativity of a potential recipient should preclude transplantation of an allograft from a seropositive donor.

As with EBV-containing AIDS–non-Hodgkin's lymphomas, the EBV genomes within PTLD generally exist as clonal circular episomes, supporting the clonality of these lesions as well as suggesting that EBV infection is an early event that occurs before clonal expansion [216]. In fact, the absence of monoclonality by EBV classification generally correlates with nonclonality by immunophenotyping and is

associated with histologic characteristics of polymorphic diffuse B-cell hyperplasia or intermediate polymorphic B-cell lymphoma. The EBV data therefore support a model in which the biologic spectrum of histologic types actually represents various stages of progression from polyclonal to monoclonal to frankly malignant lymphoproliferations.

The normal containment of EBV-infected B lymphocytes by EBV-specific cytotoxic T cells may also be abrogated by immunosuppressive agents used in organ transplant recipients. EBV may also affect the immune system by modulating levels of key cytokines such as serum IL-10, which is frequently upregulated in association with posttransplant lymphomas, especially those in which EBV is detected, following primary or reactivated EBV infection [217].

EBV latent gene expression is restricted in the PTLD lesions of solid organ transplant recipients. Using Southern blot, Western blot, and immunofluorescence analysis, Cen et al. observed a variety of restriction patterns, reflecting universal expression of EBNA-1, expression of LMP-1 in almost all cases, and only occasional expression of EBNA-2 [218]. In other studies, expression of the EBV-derived B-cell activating proteins EBNA-2 and LMP-1, as well as of their target cellular surface genes, CD23, intercellular adhesion molecule-1 (ICAM-1), and leukocyte function-associated antigen-3 (LFA-3), has been demonstrated by immunofluorescence in infiltrating B lymphocytes from bone marrow transplant recipients with EBV-related PTLD [219].

While there is a strong link between EBV positivity and PTLD, EBV-negative cases do occur. For example, Leblond et al. failed to detect EBV in 31% of the 19 B-cell PTLDs examined [193]. These EBV-negative PTLDs were more often morphologically monomorphic, and, interestingly, all but one of these lymphomas arose late, 1 year or more after transplant. These data suggest that the development of EBV-negative PTLDs in organ transplant recipients may be distinct entities. Further supporting this, EBV-negative PTLD has been found to share many cytogenetic abnormalities and copy number alteration lymphomas occurring in immunocompetent hosts [205]. Despite these differences, EBV status does not seem to be prognostic or predictive of outcome. [220].

Treatment

There is no standard treatment for PTLD given the pathologic and clinical heterogeneity. Reduction of immune suppression has been a critical component of treatment, though responses are typically durable in only 10–20% of cases [67, 221, 222]. The type of organ transplanted also dictates whether this is a feasible option. Similarly, clinical features, such as high LDH, late presentation, extranodal disease, older age, multi-organ involvement, and B symptom, often suggest a poorer response to reduction in immune suppression alone [161, 223].

In an effort to alleviate the confusion regarding therapy for PTLDs, Hanto et al. [173] categorized the posttransplant lymphomas into three histologic groups; a distinct therapeutic approach is recommended for each. For group 1, consisting of polyclonal polymorphic diffuse B-cell hyperplasia, acyclovir has successfully reversed the lymphoproliferation and is recommended with or without a reduction in immunosuppression. In group 2, the polyclonal B-cell lymphomas run the risk of progressing from polyclonal to monoclonal B-cell proliferations. Therefore, they should be treated by reduction or cessation of immunosuppression in most kidney transplant patients, allowing rejection to occur; the parallel approach in heart, heart-lung, and liver recipients, where allograft rejection would be fatal, involves acyclovir therapy and a modest reduction in immunosuppression. Finally, for group 3, the morphologically malignant monoclonal B-cell lymphomas, recommendations include standard chemotherapy or radiation therapy, combined with discontinuation of or reduction in immunosuppression. Combinations of all three modalities, acyclovir, reduction of immunosuppression, and intensive multi-agent chemotherapy, have been used successfully to treat frankly malignant lymphomas in the transplant setting.

Studies investigating the role of single agent rituximab have shown response rates of 34–42% [224–226]. A large prospective study also showed efficacy with rituximab treatment followed by CHOP chemotherapy [227]. There is also ongoing evaluation of the role of risk stratified sequential therapy with either additional rituximab or R-CHOP following rituximab depending on response [161, 227].

Lymphomas in the Setting of Bone Marrow Transplantation

Epidemiology/Demographics of Lymphomas Associated with Bone Marrow Transplants

The risk of cancer among recipients of allogeneic bone marrow transplants (BMT) is 3.8–6.7 times higher than that in the general population [229, 230]. The incidence of LPD in this treatment group ranges from 0.3 to 1.8% in different studies, generally less than the risk observed following transplants of various solid organs [230, 231]. In an overview of the allogeneic BMT experience at 235 centers worldwide reporting to the International Bone Marrow Transplant Registry (IBMTR), the cumulative incidence was 1.0 ± 0.3% at 10 years [232].

Post-bone marrow transplant lymphoproliferative disease (PBMT-LPD) develops earlier (median, 3 months) following transplant than do solid tumors of epithelial origin (median,

99 months) [168, 233]. The highest incidence in the IBMTR overview occurred 1–5 months posttransplant, with a peak at 3 months. This early onset disease (less than 1 year post-transplant) was followed by a gradual but steep decline among patients surviving for a year or more, although the risk of late-onset PBMT-LPD remained significantly higher than expected in the general population [232]. Although most reports of PBMT-LPD pertain to allogeneic transplants, lymphomas have been observed following both autologous and cord blood BMTs [234].

A variety of risk factors for the development of PTLD-LPD have been identified. Predisposition to PBMT-LPD occurs following treatment of acute GVHD with ATG or total-body irradiation and particularly, as discussed previously for solid organ transplants, with CD3-specific monoclonal antibodies [168, 214, 235]. In addition, HLA mismatch between donor and recipient, presumably because of the prolonged antigenic stimulation, contributes to a lesser degree [236]. T-cell depletion of the graft, which may result in increased susceptibility to EBV infection, has also been shown to predispose to PTLD. An identifiable risk factor late-onset PBMT-LPD was extensive chronic GVHD, possibly as a result of long-term treatment with immunosuppressive drugs.

In some studies, the incidence of PBMT-LPD varied with the underlying disease, e.g., 0.6% for treatment of hematologic malignancy versus 0.3% following transplantation for aplastic anemia [230, 237]. The risk for PBMT-LPD is particularly high among patients transplanted for congenital immunodeficiency diseases (see discussion to come). Conversely, no significant association of disease for which the transplant was performed with the risk of PBMT-LPD was seen in the IBMTR study [232].

Clinical Features and Histopathology

Histologically PBMT lymphomas resemble those seen after solid organ transplant, encompassing a large spectrum of morphologic characteristics, ranging from polymorphic B-cell hyperplasia to polymorphic B-cell lymphoma to large-cell immunoblastic lymphoma [206, 237, 238]. They are generally B-cell neoplasms, although T-cell lesions may occur [229, 238]. Interestingly, the two T-cell lymphomas observed in the IBMTR study occurred among the less frequent late-onset cases [232]. Staining for Igs has revealed both polyclonal and monoclonal lesions, although Ig heavy chain gene rearrangements have occasionally been detected within the polyclonal lesions, suggesting the presence of clonal proliferations [237]. Nonidentical monoclonal populations have been observed in separate lesions in the same patient, indicating distinct clonal derivations at the two sites [216, 239].Among PBMT-LPDs, most lymphomas have proved to be of donor origin, with host-derived lymphomas being only rarely observed [168]. Similar to PTLDs following solid organ transplants, the sites of involvement are varied and are often extranodal, involving abdominal and thoracic nodes as well as liver, spleen, gastrointestinal tract, and lungs [237].

Pathogenesis: Mechanisms Underlying Lymphomagenesis in Association with Bone Marrow Transplant

The histologic spectrum observed among PBMT-LPDs suggests the usual multistep progression from antigenically stimulated polyclonal proliferation through genetic alteration and ultimately selective expansion of a malignant clone. Analogous to the situation described for solid organ transplant recipients, potential factors operating in the context of BMT to promote tumorigenesis include the underlying disease, foreign antigenic stimuli such as mismatched HLA antigens and EBV, and the institution of immunosuppressive therapies designed to prevent GVHD. EBV DNA is almost always detected in the lymphoproliferative lesions associated with both BMT and organ transplant [206, 232, 238]. Despite the resemblance of post-BMT and post-solid organ transplant lymphomas with respect to the EBV positivity of their tumor tissue, BMT patients with PTLD fail to mount specific antibody responses to EBV VCA, early antigen, and EBNA. In the setting of BMT, a rapid increase in EBV DNA has been observed within 1–3 months after transplant, paralleling the timing of the peak of EBV-infected lymphocytes in pediatric solid organ transplant patients [240, 241]. Proposed sources of EBV in the BMT population include latently infected donor B lymphocytes, latently infected host B lymphocytes, the host nasopharynx, and blood transfusions. Since most cases of PBMT-LPD are of donor origin, the most likely etiology of EBV infection is via infected B lymphocytes transferred from the donor at the time of transplant or by infection of donor lymphocytes following transplantation into the host [237].

In addition to the clustering of EBV-negative lymphomas among late-onset cases, the two T-cell lymphomas observed in the IBMTR study occurred in the late-onset group [232]. This brings to mind the few T-cell lymphomas seen among PTLDs observed by Leblond et al. following solid organ transplantation, which also were of late onset and also tended to be EBV negative [193]. As pointed out by these authors, these data suggest that the development of EBV-negative PTLDs in organ transplant recipients may represent a morphologically and clinically distinct entity, depending on the accumulation of additional genetic changes and increasing in number with time after transplant.

Treatment of Post-Bone Marrow Transplant Lymphomas

Limited information is available regarding the treatment of PBMT-LPD. Although a strong association exists between EBV and these lymphomas, the data suggesting efficacy of acyclovir or ganciclovir are confined to a small number of cases, and do not support the use of these antiviral agents in either a prophylactic or a therapeutic mode in the setting of bone marrow transplants [237, 242, 243]. Based on the association of PBMT-LPDs with EBV and T-cell depletion of the allograft, together with their tendency to be of donor origin, patients who developed EBV-related lymphoma after receiving T-cell-depleted marrow were infused unirradiated donor lymphocytes [244]. The underlying hypothesis that the infused cell populations would supply missing cytotoxic T-cell precursors presensitized to EBV in the donor's microenvironment, which might control the proliferation of EBV-transformed donor cells in the host, was supported by observed responses in this small set of patients. An additional therapeutic approach has utilized interferon-α which, together with intravenous γ-globulin, has elicited responses in hematological malignancies complicating BMT [206, 243]. Finally, the use of CD21- and CD24-specific B-cell antibodies in patients with severe PBMT-LPD led to complete responses in 16 of 28 patients (57%), similar to the results with organ transplant patients, among whom 20 of 31 had complete responses (64%) [245, 246]. However, only 35% of bone marrow transplant patients survived more than 1 year, significantly less than the 55% survival among solid organ transplant recipients. The CD20-specific B-cell monoclonal antibody rituximab has also been used successfully to induce complete responses in three patients who developed EBV-associated lymphomas following stem cell transplantation [245]. By analogy to the solid organ transplant setting, the absence of firmly established effective treatments for PBMT-LPD, together with the association of this disease entity with EBV infection, has led some to recommend avoiding exposure of seronegative recipients to this virus and to select an EBV seronegative donor whenever possible for such transplants.

Lymphomas in Specific Autoimmune Diseases

The rheumatologic, or autoimmune, disorders offer another disease context in which an increased incidence of LPD is observed. The underlying mechanisms in these disease entities involve dysregulation leading to hyperactivity of the immune system.

The intimate relationship between malignancy and autoimmunity is particularly evident in Sjögren's syndrome, in which a classic histopathologic feature is the benign lymphoepithelial lesion, an infiltration of glandular tissue by B and T lymphocytes and plasma cells. Non-Hodgkin's lymphomas occur in Sjögren's syndrome patients with a frequency between 12 and 43 times that seen in the general population [247–249]. The lymphoproliferative lesions of the syndrome range from benign pleiomorphic lymphoid infiltrates to morphologically malignant lymphoma, resembling the spectrum of lesions described in transplant recipients [250]. The term *pseudolymphoma* refers to the middle portion of this spectrum and applies to those lesions that contain tumor-like aggregates of lymphoid cells but fail to meet the histologic criteria for malignancy [250]. These lesions may be localized to the salivary gland or appear as extraglandular lymphoid infiltrates.

All the morphologically malignant lymphomas are B cell in origin and diffuse in pattern of growth. The predominant histologic types include large cell, particularly immunoblastic, mixed large and small cell, and even small-cell lymphomas, with marked histologic variation within individual tumors. These are probably all lymphomas of the MALT type, similar to gastric MALT lymphoma [251]. The antigenic stimulus within the salivary gland has not been defined. Loss of intracytoplasmic Ig has been noted in association with terminal lymphomas, suggesting dedifferentiation of plasma cells and immunoblasts. Progression from a polyclonal benign lymphoproliferative lesion to monoclonal lymphoma has also been demonstrated. In other studies, however, clonal Ig gene rearrangements have been detected in presumably benign lymphocytic infiltrates [252]. A possible explanation for this apparent inconsistency is that the clonal expansion is due to a generalized defect in immune surveillance that characterizes Sjögren's syndrome, whereas the development of lymphoma reflects additional genetic alterations that promote neoplastic transformation [249].

Accompanying immunologic abnormalities include hypergammaglobulinemia, a high incidence of autoantibodies, organ-specific (anti-salivary duct) autoantibody, and a local excess synthesis of Igs, especially IgM macroglobulins [198]. While increased IgM levels in involved tissues or in serum are suggestive of concurrent Waldenstrom's macroglobulinemia, a low IgM level may herald the appearance of malignant lymphoma and is a poor prognostic sign. A fall in rheumatoid factor may accompany the decrease in IgM, leading to generalized hypogammaglobulinemia and loss of autoantibodies concomitant with the development of lymphoma [198]. The search for a potential viral role in lymphomagenesis associated with Sjögren's syndrome has been limited. However, based on a small number of cases, in which the EBV-encoded small RNA EBER-1 was not expressed in the benign lymphoproliferations of Sjögren's syndrome, EBV infection does not appear to play a key role in the pathogenesis of these lesions [253]. HHV-8 DNA

sequences were detected in a single bilateral MALT lymphoma of the parotid gland associated with Sjögren's syndrome, in contrast to 53 other histopathologic types of salivary gland tumors, but further investigation is required to establish a true pathogenetic association between this virus and the lymphoma [254].

The prognosis of patients with Sjögren's syndrome and LPD is guarded. In some cases, however, therapy with radiation or immunosuppressive agents such as cyclophosphamide, chlorambucil, and prednisone has led to improvement of the primary disease symptoms of xerostomia and conjunctivitis sicca as well as eliciting a diminution in tumor size. Series employing more recent treatment approaches have not been reported.

A variety of lymphoid neoplasms have been noted in individuals with antecedent rheumatoid arthritis and, to a lesser extent, systemic lupus erythematosus (SLE). However, statistical analysis has not consistently proven an increased susceptibility to LPD in these disease states. Evidence from a number of studies suggests that the incidence of cancer among individuals with rheumatoid arthritis ranges from 0.6 to 4.1% [255–258]. Other studies have demonstrated no overall increase, and even a lower incidence, of malignancies in patients with rheumatoid arthritis [255, 256]. The increased risk of lymphoma in association with rheumatoid arthritis is generally reported as modest, only 2–2.5 times that in the general population, although relative risks as high as 24 have been noted [259–261]. Nevertheless, within the 1–2% of rheumatoid arthritis patients who have Felty syndrome (rheumatoid arthritis with neutropenia, splenomegaly, and recurrent infections), the incidence of all cancers is twice that in the control population. The risk of non-Hodgkin's lymphoma in Felty syndrome is increased 12-fold, approximating the increased risk of non-Hodgkin's lymphoma in Sjögren's syndrome, a condition that is sometimes associated with Felty syndrome [247]. The susceptibility of Felty syndrome patients to non-Hodgkin's lymphoma probably derives from their particular array of immunologic alterations, which include bone marrow depression and an increased frequency of the HLA-DQ haplotype, linked to control of suppressor T-cell function. In general, however, rheumatoid arthritis is associated with only minimal immunologic deficits, including failure of PMBCs to respond to recall antigens, a moderate decrease in both B and T cells, and a decrease in CD8 cells, accompanied by an increase in both CD4 cells and the CD4:CD8 ratio [262]. A reduction in natural killer cell activity, observed in some studies in the synovial fluid and whole blood of patients with rheumatoid arthritis, has been suggested as a possible explanation for the increased tendency to develop non-Hodgkin's lymphomas [261]. EBV DNA sequences have been demonstrated in rheumatoid arthritis-associated lymphoma, 80% being type 1 [263]. In contrast to Sjögren's syndrome, there is only a rare association of MALT lymphoma with rheumatoid arthritis.

A positive association between SLE and non-Hodgkin's lymphoma has been observed in some studies. Lewis et al. noted a 4% incidence of cancer among patients with SLE, which is over twice the frequency in their controls [255]. This contrasts with their data on rheumatoid arthritis, in which cancer risk was not increased. Among cancers, lymphomas appear to have the strongest association with SLE [264]. Nevertheless, the question of a genuinely increased risk of cancer, and specifically of LPD, in patients with SLE remains unanswered.

The skin is included in the spectrum of tissues affected by a number of the connective tissue diseases, such as SLE, dermatomyositis, relapsing polychondritis, and lichen sclerosus et atrophicus. Atypical lymphoid infiltrates arising in such autoimmune cutaneous lesions have been categorized as either pseudolymphomas or malignant lymphomas based on a combination of clinical, histologic, immunologic, and genotypic attributes.

Primary non-Hodgkin's lymphoma of the thyroid gland accounts for 5% of thyroid malignancies and less than 2% of extranodal lymphomas [265]. These generally occur in the context of chronic lymphocytic thyroiditis (Hashimoto's disease). The male-to-female ratio in one study was 1:4, with mean ages of 63 and 73 years, respectively [266]. Thus, patients typically are middle-aged females who are euthyroid, lack antithyroid antibodies, and present with a cold nodule in the neck. Most cases are localized, stage IE or IIE. In view of their tendency to remain localized for prolonged periods with late, distant relapses, the thyroid lymphomas, along with those of the breast, lung, parotid, and gastrointestinal tract, are believed to belong the subset of extranodal lymphomas, designated MALT lymphoma [267]. The thyroid antigen that serves as the stimulus is not defined. As in other settings of immunodeficiency, the lymphomas occurring in the thyroid are primarily of the diffuse large-cell type, especially with immunoblastic morphology, although some of the tumors are small lymphocytic in histology.

Histologic differentiation of diffuse chronic lymphocytic thyroiditis from malignant lymphoma may be difficult, recalling the spectrum of histologies observed among the lymphomas found in Sjögren's syndrome, transplant recipients, and other immunocompromised states. Areas of chronic lymphocytic thyroiditis are sometimes present in residual thyroid tissue adjacent to areas of malignant lymphoma, suggesting progression through this spectrum from benign hyperplasia to frankly malignant lesions. The polyclonality together with the predominantly T-cell nature of the lymphoid infiltrates of thyroiditis presents a stark contrast to the monoclonal B cells that make up MALT-type lymphomas, including those of the thyroid [268]. However, the emergence of a monoclonal B-cell proliferation in the midst of a

polyclonal B- and T-cell inflammatory response appears to be a common mechanism for MALT lymphomas involving different organs. Only rarely have T-cell thyroid lymphomas been observed. A role for EBV in promoting the progression from Hashimoto's thyroiditis to malignant lymphoma is suggested by the observation of a small subset of thyroid lymphomas in which strong and homogeneous signals for EBER appeared in the nucleus of most cells by both in situ hybridization and immunohistochemistry [269]. However, larger numbers and demonstration of EBV clonality in tumor cells are necessary before EBV can be definitively regarded as a contributor to lymphomagenesis in the thyroid.

Treatment in early studies of thyroiditis-associated lymphomas consisted of local modalities only, first surgery and later radiation, the latter yielding an overall relapse rate of 37.1%, primarily as distant relapse (30.8%) [265]. Given this outcome, combined modality therapy, which has an overall relapse rate of 7.7% (5.1% distant relapse), is now being espoused as the optimal approach to thyroid lymphoma [265]. Overall, the outcome in more recent studies has been favorable following appropriate therapy.

Autoimmune diseases of the gastrointestinal tract have also been associated with an increased incidence of lymphomas. The risk of malignant lymphoma is elevated in individuals with adult celiac disease (celiac sprue, gluten-sensitive enteropathy, and nontropical sprue), an autoimmune disease in which infiltration of the lamina propria by plasma cells and lymphocytes is believed to mediate damage to the intestinal mucosa that results in villous atrophy and malabsorption. The lymphomas that occur in association with celiac disease represent a specific subtype of primary intestinal T-cell lymphoma, *enteropathy-associated T-cell lymphoma*, confirmed as monoclonal by TCR gene rearrangement [270]. Despite their T-cell origin, they may be somewhat dedifferentiated in immunophenotype [271]. The few B-cell lymphomas that are seen are usually diffuse large-cell or immunoblastic lymphomas [272]. Enteropathy-associated T-cell lymphomas commonly occur in the jejunum, often in areas of ulcerative jejunitis, and they tend to affect patients with a short history of adult celiac disease, rather than those whose disease dates back to childhood [137]. The prognosis is poor, reflecting, in part, late stage at diagnosis and poor performance status. Nevertheless, some evidence exists to suggest that these lymphomas may be prevented by adherence to a strictly gluten-free diet.

Other gastrointestinal disease settings that may predispose to primary small intestinal malignant lymphomas include inflammatory bowel disease, both ulcerative colitis and regional enteritis (Crohn's disease) and Mediterranean α chain disease. Colonic lymphoma has rarely been observed superimposed on chronic ulcerative colitis. One report describes the occurrence of reversible EBV-related polymorphic B-cell lymphoproliferation in a patient with Crohn's disease during treatment with azathioprine therapy, resembling the lymphomas that are observed in transplant recipients [273]. The immunoproliferative small intestinal disease that occurs in α chain disease is a low-grade subtype of MALT lymphoma (see previous discussion), except that plasma cells secreting large amounts of α heavy chain without light chain are prominent in the intestine and mesenteric lymph nodes [137].

Pathogenesis: Mechanistic Basis for Autoimmune Disease-Associated Lymphomagenesis

The immune dysregulation that is inherent in patients with autoimmune diseases is believed to contribute to their tendency to develop lymphoproliferative malignancies. Hypergammaglobulinemia with a high incidence of autoantibodies and excessive local synthesis of Igs in some cases may provide chronic intense stimulation of B-cell proliferation [250]. Superimposed on this, an impaired immune surveillance may allow uncontrolled proliferation of abnormal cells. Lastly, as is the case for patients with PTLD, use of immunosuppressive agents may contribute to tumorigenesis in autoimmune disease. Cyclosporine, azathioprine, and methotrexate, common immune suppressive agents used in autoimmune disease, have been linked to the development of lymphoma [274–276]. More recently, given the growing popularity of anti-TNF (either soluble receptor or monoclonal antibody) agents, there has also been increased attention regarding a possible link to malignancy as well.

TNF-alpha was identified in 1975 as factor released by host cells that mediated endotoxin-induced tumor necrosis [277]. While subsequent studies have not clearly identified TNF-alpha as an anti-cancer agent, it has been found to have other critical affects on immune function. Specifically, TNF-alpha stimulates macrophage activation, monocyte differentiation, neutrophil recruitment, and granuloma formation [278, 279]. Therefore, targeting this proinflammatory cytokine has been an effective strategy in a variety of inflammatory illnesses, such as rheumatoid arthritis, psoriasis, ankylosing spondylitis, and inflammatory bowel disease [228, 280–282].

Five inhibitors, infliximab, adalimumab, certolizumab pegol, golimumab, and etanercept, have been developed and approved by the US Food and Drug administration. The first drug developed was infliximab, a chimeric mouse/human monoclonal antibody. In an effort to improve tolerability, adalimumab and golimumab, fully humanized monoclonal antibodies, as well as certolizumab, a humanized antibody to the Fab fragment of TNF-alpha monoclonal antibody were developed. Etanercept, a recombinant protein composed of the TNF receptor and the Fc portion of the human IgG1, blocks TNF interaction with cell surface receptors.

Since the advent of these drugs, there has been concern regarding an increased susceptibility to malignancies, specifically to lymphoma. Postmarket adverse event surveillance by the FDA revealed 26 cases of lymphoproliferative disorders following treatment with infliximab or etanercept. In two instances, lymphoma regression was seen following discontinuation of the anti-TNF therapy [283]. Subsequently, a meta-analysis of trials including over 3000 patients demonstrated a dose-dependent increased risk of malignancy in patients with rheumatoid arthritis using anti-TNF-alpha therapy [284]. While this data suggests an increased risk of malignancy, other similar studies have shown conflicting data with no clear association between TNF-alpha inhibitor use and malignancy [285, 286]. In regards to lymphoma specifically, results are confounded by the fact that patients with more severe autoimmune disease are more likely to use this therapy than patients with mild disease. However, studies have been performed that do not demonstrate a clear risk of lymphoma as compared to patients using alternative immunomodulatory drugs [287, 288].

The interaction of specific immunodeficiencies in individuals with rheumatoid arthritis with infection by EBV may also predispose toward malignancy. Persistent EBV infection has been documented in a patient with rheumatoid arthritis and a diffuse large-cell lymphoma [262]. EBV DNA, RNA, and LMP have also been detected in malignant cells in patients with autoimmune disease [289, 290]. Furthermore, although B cells from EBV-immune patients with rheumatoid arthritis respond appropriately to EBV stimulation by increasing Ig production, their T cells are deficient in mediating late suppression of this Ig production by the autologous EBV-infected B cells [291].

Lymphomas in the Setting of Congenital Immunodeficiency Diseases

The primary immunodeficiency diseases (CID), i.e., diseases that are either congenital or genetically determined, are rare conditions, seven of which account for 95% of cases (Table 47.3). Among individuals with CIDs, there is an increased risk of malignancy, with the overall cancer incidence in the immunodeficient population varying between 2 and 25% [201, 292]. Although each of the CIDs is associated with a particular incidence and constellation of malignancies, over 50% of the cancers are lymphoid neoplasms, substantially higher than that in the general population [293]. Of these, the majority are non-Hodgkin's B-cell lymphomas, although, in contrast to most other immunodeficiency settings, Hodgkin's disease is also well represented [294]. T-cell lymphoma has only rarely been reported. The non-Hodgkin's lymphomas are clearly the most common neoplasms in five of the seven major immunodeficiency states: Wiskott-Aldrich syndrome (80%), IgM deficiency (56%), ataxia-telangiectasia (46%), severe combined immunodeficiency (74%), and common variable immunodeficiency (46%) [201]. These lymphomas make up a large proportion of the cancers in the other two major CIDs, X-linked (Bruton's) hypogammaglobulinemia (33%) and IgA deficiency (16%). There are even reports of lymphomas in association with rarer CIDs, including B-cell lymphomas in Di George syndrome and Nijmegen breakage syndrome [295–297]. The proportion of non-Hodgkin's lymphomas among cancers (65%) is the same among both adults and children with CID, unlike the general population, in which only 13.5% of pediatric cancers are non-Hodgkin's lymphomas, while leukemias represent 48–76% of cancers in this age group [293].

Clinical Features and Histopathology

The non-Hodgkin's lymphomas are virtually all diffuse. As in the settings of immunodeficiency previously described, the large-cell lymphomas, particularly the large-cell immunoblastic lymphomas, constitute a larger percentage (38%) than in the general population (4.2%), whereas Hodgkin's lymphoma and the small-cell lymphomas are underrepresented [293, 298, 299].

Noteworthy differences exist in the distribution of histological types among the different CIDs. Whereas the relative frequencies of the three histological types of lymphoma (diffuse large-cell, small-cell, and Hodgkin's lymphoma) are similar among patients with ataxia-telangiectasia, combined variable immunodeficiency, and IgA deficiency, IgM-deficient patients exhibit a higher percentage of large-cell lymphomas [300]. This is also true of individuals with Wiskott-Aldrich syndrome, which, interestingly, is associated with impaired IgM responses [301]. A comparison of ataxia-telangiectasia and Wiskott-Aldrich syndrome reveals additional important differences. Patients with the former disease regularly develop both non-Hodgkin's lymphomas and Hodgkin's lymphoma, in contrast to individuals with the latter disease who present with Hodgkin's disease far less often [299–301]. Furthermore, the predominant subtype of non-Hodgkin's lymphoma in ataxia-telangiectasia patients is diffuse large B-cell lymphoma, whereas two-thirds of the non-Hodgkin's lymphomas among Wiscott-Aldrich syndrome patients have a large-cell immunoblastic histology. It is also of interest that among individuals with ataxia-telangiectasia, 50% of the Hodgkin's lymphoma is of the lymphocyte-depleted subtype, and it can occur in very young patients. This contrasts sharply with the general population where lymphocyte-depleted Hodgkin's lymphoma is diagnosed in fewer than 5% of cases and tends to occur in elderly men with advanced stage disease or at relapse after treatment of other histologies [109, 113].

Table 47.3 Congenital immunodeficiency diseases

Immunodeficiency disease	Clinical features/Immune deficiencies	Genetics/epidemiology	% of cancers that are lymphomas, types of cancers, with statistics	Association with EBV	References
Ataxia-telangiectasia (AT)	Cerebellar ataxia, oculocutaneous telangiectasia, recurrent sinopulmonary infections	Autosomal recessive	– Lymphomas—60% of cancers, usually T cell	++	[751, 821, 822, 824, 825, 829, 835, 843–854, 860, 868, 869, 882–884, 939]
	Deficiencies of IgA, IgE, abnormal T-cell-mediated responses, hypoplastic thymus glands	*ATM* (ataxia telangiectasia mutated) gene at chromosome 11q23	– Hodgkin's disease—11% of cancers		
	Defective excision repair of irradiation-induced double-stranded DNA breaks; increased intrachromosomal recombination	Somatic genetic alterations	– Acute leukemia—21% of cancers		
	α-fetoprotein levels elevated	t(14;14) (q11;q32.1)inv.(14) (q11q32),t(x;14),inv.(7) (p13;q35),t(7;7) (p13;q35),t(7,14) (p13;q11),t(7;14)(q13;q11) rarely t(X;14) (q28;q11)	– B-cell lymphoma—12% of AT patients		
		Prevalence: 1:200,000	– Breast cancer increased 3.9-fold in female relatives of AT patients		
		Birth frequency: 1 per 300,000 births	– 13.8% of sporadic breast cancers due to *ATM* mutation		
		ATM mutation carriers: 1% of U.S. population			
Wiskott-Aldrich syndrome (WAS)	Eczema, thrombocytopenia, susceptibility to infections	Sex-linked	– B-cell lymphomas 80% of cancers	+	[751, 822, 825, 829, 838, 840, 842, 870–873, 885]
	Combined B- and T-cell variable immunodeficiency, low IgM, increased IgA and IgE	*WASP* (Wiskott-Aldrich syndrome protein) gene at	– Hodgkin's disease—4% of cancers		
		Chromosome Xp11.22–11.23			
		Birth frequency: 4 per million live male births			
Severe combined immunodeficiency (SCID)	Severe infections beginning in neonatal period	Autosomal recessive or sex linked	Lymphomas B 55–74% of cancers	+	[751, 822, 825, 829]
	Defective stem-cell differentiation to T and B cells, some with adenosine deaminase deficiency		Hodgkin's disease B 9.5% of cancers		
			Acute leukemia		

Common variable immunodeficiency (CVI)	Encompasses several syndromes: Recurrent bacterial infections of the respiratory tract, sinusitis, otitis media, bronchitis, pneumonia due mostly to encapsulated bacteria (*Streptococcus pneumoniae*, *Hemophilus influenzae*)	Autosomal dominant with incomplete penetrance	Lymphomas—49% of cancers	+	[408, 751, 822, 825, 826, 829, 832, 841, 886]
	Normal numbers of B cells with hypogammaglobulinemia, deficits of T-cell immunity in some patients, cutaneous anergy	Associated with mutations of C4-A and C2 gene alleles in the class III MHC region on chromosome 6	Hodgkin's disease—7% of cancers		
			CLL		
			Peripheral T-cell lymphoma—1 case		
			Benign lymphoproliferative disorders: 30% of CVI patients		
			Lymphomas—Increased 30-fold over general population; female CVI patients B increased 100-fold		
IgM deficiency (isolated, selective)	Upper respiratory infections, especially gram-negative bacteria and bacteremia	Frequency: 1/1000 persons	Lymphomas—71% of cancers	NA	[822, 829]
IgA deficiency (isolated, selective)	Recurrent infections, often with autoimmune disease and GI disorders, or asymptomatic	Frequency: 1/500 to 1/700 persons	– Lymphomas B 16–31% of cancers,	NA	[751, 822, 825, 829, 831, 837]
			– Hodgkin's disease—8% of cancers		
			– Gastric adenocarcinoma		
			– Abdominal T-cell lymphoma B 1 case		
X-linked (Bruton's)	Infections with encapsulated organisms (*Hemophilus influenzae*, *Pseudomonas aeruginosa*, *Diplococcus pneumoniae*);	Sex-linked *BTK* (Bruton's tyrosine kinase) gene at	– Lymphomas—33% of cancers	B (no EBV receptors)	[587, 822–824, 826, 829, 841, 874, 940]
Hypogammaglobulinemia/ Agammaglobulinemia	B-cell maturation defect B few B lymphocytes, no Ig-secreting plasma cells	Chromosome Xq22	– Acute lymphocytic leukemia		
			– Chronic myelogenous leukemia		

(continued)

Table 47.3 (continued)

Immunodeficiency disease	Clinical features/Immune deficiencies	Genetics/epidemiology	% of cancers that are lymphomas, types of cancers, with statistics	Association with EBV	References
X-linked	Chronic or fatal infectious mononucleosis with hepatic necrosis	Sex-linked	– B-cell lymphoma	+++	[3, 34, 160, 250, 824, 875–879, 881, 882, 887]
Lymphoproliferative disease (XLP; Duncan's disease)	Variable hypogammaglobulinemia, decreased NK-cell activity, abnormal CD4/CD8 ratios, decreased memory T-cell activity against Epstein–Barr virus	*DSHP/SH2D1A/XLP* gene at	– 35% of males with XLP		
	Induction of *DSHP/SH2D1A* may interfere with inhibition of lymphocyte activation	Chromosome Xq25			
		DSHP/SH2D1A mutated in 70% XLP patients			
Bloom's syndrome	Telangiectatic erythema, stunted growth	Autosomal recessive	– Increased incidence of non-Hodgkin's lymphomas, acute lymphocytic leukemias, solid tumors	NA	[1, 849, 863, 864, 866, 867]
	Decreased immunoglobulins of one or more classes, poor lymphocyte response to certain mitogens	*BLM* (Bloom's) gene at	– Non-Hodgkin's lymphomas—21% of cancers		
	Chromosome breakage and rearrangements, especially quadriradial (Qr) cytogenetic abnormality, usually chromosome 1	Chromosome 15q26.1			
	Increased sensitivity to ultraviolet radiation				
Xeroderma pigmentosum	Skin atrophy, photosensitive dermatitis	Autosomal recessive	– Increased incidence of skin cancer	NA	[1]
	Mild immunodeficiency				
	Defective DNA repair following exposure to ultraviolet light				
Nijmegen breakage syndrome	Small stature, severe microcephaly, bird-like facial appearance, progressive decrease of intellectual function	Autosomal	– 40% of patients develop cancers	NA	[835, 836, 849]
	Deficiency in B- and T-cell function	*Nibrin* gene at	– Leukemias and lymphomas B 85% of cancers		
	Frequent infections, especially paranasal sinuses, lower respiratory tract	Chromosome 8q21	– B-cell lymphomas B most common		
	Chromosomal breakage syndrome, chromosomal hypersensitivity to X-rays, radioresistance of DNA replication				

Fanconi's anemia	Progressive pancytopenia, diverse congenital abnormalities	Autosomal recessive	– Increased incidence of acute myeloid leukemia	NA	[827, 833]
	Mild immunodeficiency	Somatic genetic alterations: t(11;14) (p13;q32)	– T-cell lymphoblastic lymphoma B 1 case		
	Hypersensitivity to the clastogenic effect of DNA cross-linking agents, such as diepoxybutane, mitomycin C				
Diamond-Blackfan anemia	Chronic pure red cell aplasia, macrocytic anemia	Congenital	– Acute myelogenous leukemia	NA	[941, 942]
	Ocular, thumb, craniofacial, genitourinary abnormalities		– Acute lymphocytic leukemia		
			– Hodgkin's disease B few		
			– Non-Hodgkin's lymphoma B 1 case		
Hyper IgM syndrome	Absence of hypoxanthine guanine phosphoribosyl transferase (HGPRT), leading to defective salvage pathway of purine biosynthesis B contributes more to B lymphocytes than T lymphocytes	Sex-linked	Lymphomas—56% of cancers	NA	[751]
	Increased numbers of B cells		– Hodgkin's disease—25% of cancers		
	Increased IgM, decreased amounts of other isotypes				
	Activated T cells have abnormal CD40 ligand, which cannot bind CD40 on B cells and thus fails to stimulate B-cell proliferation and IgE secretion				

NA not addressed

Unlike the large-cell immunoblastic lymphomas that occur in the general population, those observed in patients with CIDs are typically extranodal and often localized. The CNS is frequently involved, as are the liver, lung, and gastrointestinal tract, resembling the non-Hodgkin's lymphomas observed in transplant recipients. Even though chronic benign lymph node enlargement frequently occurs in patients with Wiskott-Aldrich syndrome, who have affected nodes displaying a spectrum of hyperplastic changes, frank lymphoma tends to occur extranodally [302].

Pathogenesis: Mechanistic Basis for Congenital Immunodeficiency-Associated Lymphomagenesis

The disrupted immunoregulation that characterizes the CIDs is believed to contribute to their association with cancer, especially lymphoid malignancies. A number of mechanistic bases for these immune defects are unique when compared with those operating in other immunodeficiency settings.

Several syndromes feature defects in DNA metabolism as well as the accumulation of chromosomal aberrations. Ataxia-telangiectasia, for example, is characterized by cells that are exquisitely sensitive to X-rays and experience excessive chromosomal breakage secondary to defective excision repair of irradiation-induced breaks in double-stranded DNA [303, 304]. The inherited mutation in individuals with ataxia-telangiectasia is in the *ATM* gene, which is located on chromosome 11q22–23 [305]. *ATM* has homology to several cell cycle checkpoint genes, suggesting that a nonfunctional *ATM* may lead to defects in the surveillance and appropriate cellular responses to DNA damage [306]. The contribution of *ATM* alterations to lymphomagenesis is supported by the observation of acquired 11q22–23 hemizygous deletions in sporadic lymphomas of various histologies (follicular, mantle-cell, and diffuse large-cell) and of *ATM* missense mutations in a high percentage of sporadic T-cell prolymphocytic leukemias and occasional B-cell non-Hodgkin's lymphomas and B-cell chronic lymphocytic leukemias [307, 308].

Rates of spontaneous intrachromosomal recombination are also increased in patients with Ataxia-telangiectasia [306]. Approximately 10% of all T lymphocytes circulating in the peripheral blood of these patients contain karyotypically abnormal T cells, typically involving chromosome 7 and 14 rearrangements at specific breakpoints (Table 47.3) [309]. The usual breakpoints, at 14q11 and 14q32.1, encompass the T-cell receptor-α (TCR-α) and IgH loci, respectively [310, 311]. Importantly, the percentage of cells containing a typical monoclonal, cytogenetic abnormality may increase over time, preceding both identifiable TCR monoclonality and an overt malignant phenotype [309, 311]. This defect appears to be unique to ataxia-telangiectasia, since T cells from other immune deficiency diseases, including common variable immunodeficiency and Wiskott-Aldrich syndrome, fail to show such an abnormal T-cell receptor ratio. The lymphomas associated with ataxia-telangiectasia are usually T cell in phenotype, but rare B-cell lymphomas have been described [312].

An additional cytogenetic abnormality, telomeric fusions, is also associated with large pre-leukemic clones. It is thought that these fusions result from the combined effect of the accelerated telomere shortening seen in cells homozygous for *ATM* mutations and the growth advantage of cells in large clones [313]. The picture that emerges is one of sequential genetic alteration, contributing to the progression toward tumorigenesis.

Bloom's syndrome is a rare CID in which cells exhibit increased sensitivity to ultraviolet radiation [314]. It is characterized by a cytogenetic rearrangement involving a quadriradial configuration (Qr), usually of chromosome 1 [315]. Patients with the disease are homozygous for a mutation in the Bloom's syndrome gene, *BLM*, located on chromosome 15q26.1 [316]. *BLM* encodes a protein that is homologous to the ReqQ DNA helicases and, when mutated, leads to genetic instability [317]. Xeroderma pigmentosum (XP) and Fanconi's anemia, both of which show abnormal DNA metabolism and mild immunodeficiency, are also associated with an increased risk of cancer, particularly leukemias and skin tumors [318]. An increased risk of cancer also exists among first-degree relatives of patients with ataxia-telangiectasia, Bloom's syndrome, and Fanconi's anemia [319].

Defects in DNA repair and recombination are not central to all of the lymphoma-associated CIDs. Wiskott-Aldrich syndrome is an X-linked recessive disease that is caused by mutations in the *WASP* gene at chromosomal locus Xp11.22, which encodes Wiskott-Aldrich syndrome protein, WASP, a protein involved in lymphoid cell signal transduction pathways and regulation of actin cytoskeleton rearrangement [320, 321]. The immune deficit in this syndrome appears to result in part from accelerated destruction of lymphocytes, marked by an increased frequency of spontaneous apoptosis, with attenuated Bcl-2 expression [320]. T cells from Wiskott-Aldrich patients show diminished proliferative responses and failure to appropriately upregulate cytokines like IL-2. These immunologic abnormalities may well contribute to the propensity to lymphomagenesis of this patient population.

EBV and Congenital Immunodeficiency Disease Act in Synergistic Fashion to Promote Lymphomagenesis

Although in a number of CIDs EBV is believed to interact synergistically with the underlying immune deficiency to

promote malignancy, this is not universal. At one extreme, individuals with Bruton's agammaglobulinemia, which involves B-cell deficiencies that are due to mutation of the X-linked Bruton's tyrosine kinase (*BTK*) gene, lack EBV receptors and therefore cannot be infected by this virus [322, 323]. By contrast, in the curious condition of X-linked lymphoproliferative syndrome (XLP), EBV has been shown to play a vital role in triggering the onset or exacerbation of immunological deficits [324].

DSHP/SH2D1A, the gene for XLP, maps to chromosome Xq25 and is mutated in approximately 70% of XLP patients (Table 47.3) [325, 326]. The protein encoded by this gene consists of a single SH2 domain that is homologous to the SH2 domain of SHIP, an inositol polyphosphate 5-phosphatase that functions as a negative regulator of lymphocyte activation [327]. Induction of *DSHP* may therefore sustain an immune response by subverting the normal inhibition of lymphocyte activation that is mediated by SHIP [328]. Overall, males carrying the allele for XLP exhibit normal cellular and humoral immunity before EBV infection. However, subtle deficits, such as an elevated CD4/CD8 ratio, a partial IgA deficiency, and failure to switch from IgM to IgG on OX174 challenge, have been documented [329]. During acute EBV infection, they develop vigorous cytotoxic cellular responses against EBV-infected and uninfected autologous target cells, including hepatocytes, often succumbing to a fatal infectious mononucleosis syndrome as a result of fulminant liver failure and hepatic necrosis or hemophagocytic syndrome [330]. Survivors of infectious mononucleosis manifest varying degrees of immunodeficiency, commonly involving hypogammaglobulinemia of different isotypes with absence of the normal post-infectious mononucleosis antibodies to EBNA and usually to VCA [331]. Among the other defects acquired following infection with EBV are loss of natural killer cell activity, abnormal CD4/CD8 ratios, and destruction of the thymic epithelium [324].

Four major pathogenetic outcomes have been described following EBV infection in males with XLP: (1) acquired agamma- or hypogammaglobulinemia with necrosis of lymph nodes in 20%; (2) aplastic anemia, including various gradations of neutropenia and red cell aplasia; (3) fatal and chronic infectious mononucleosis in almost two-thirds of patients, 85% proving fatal; and (4) malignant B-cell lymphoma in about one-third of males with XLP. XLP patients with lymphoma may manifest concomitant hypogammaglobulinemia or fatal infectious mononucleosis, or both [330].

Interestingly, 78% of the primary lymphomas in XLP patients occur in the intestine, frequently the ileocecal region [330]. Most are localized stage I and II tumors. Histologically, Burkitt's lymphomas constitute the largest group (45%), but large cell, immunoblastic, and other types have also been seen. Because of the intimate association between EBV infection and the immunodeficiency observed in XLP, this disease entity has served as a prototype for understanding the interaction of these factors in the development of non-Hodgkin's lymphoma, a frequent outcome. In addition to precipitating or exacerbating a preexisting subtle immunodeficiency in XLP patients, EBV stimulates polyclonal B-cell proliferation. The EBV-suppressed immune surveillance that characterizes XLP is believed to allow such polyclonal cell growth to proceed unchecked, providing more raw material for subsequent genetic mutations that ultimately might lead to malignancy. Despite the overwhelming evidence for intricate synergism between the inherited immunologic abnormality and EBV in inducing lymphomagenesis in XLP patients, rare cases have been reported of boys with documented mutations in the *DSHP/SH2DA1* gene developing non-Hodgkin's lymphomas in the absence of EBV infection [332].

EBV genomes have also been identified in lymphomas associated with ataxia-telangiectasia. Furthermore, patients with this disease frequently display abnormal antibody responses to EBV, characterized by high EBV antibody titers to VCA, an increased incidence of antibodies to early antigen, but low or absent antibodies to EBNA [333, 334]. The immunological basis for such antibody expression undoubtedly contributes to the tendency to develop lymphomas, as in the case of XLP. The immunological dysregulation may, in turn, result from and/or cooperate in a synergistic manner with the DNA repair and chromosomal recombination abnormalities of ataxia-telangiectasia to promote malignant transformation.

Interestingly, EBV DNA has also occasionally been detected in monoclonal B cells from the tumor tissue of malignant lymphomas in the setting of other CIDs such as Wiskott-Aldrich syndrome and common variable immunodeficiency [335, 336].

Treatment of Congenital Immunodeficiency Disease-Associated Lymphomas

Information on treatment strategies addressed specifically to lymphomas that occur in the setting of CIDs is sparse. The use of bone marrow transplantation to reconstitute the immune deficiency fully has been proposed as a means of preventing the development of lymphomas [337]. In patients with XLP, EBV-induced malignant transformation is the result of uncontrolled expansion of infected B cells and proliferation of activated T cells. Therefore, rituximab has been used to control acute EBV infection by eliminating infected cells and preventing subsequent lymphomas [338]. Antiviral agents such as acyclovir could theoretically also decrease viral load, though clinical trials have not been performed.

Radiation therapy and surgery have also been attempted in some cases. Whereas treatment of lymphomas is often successful in settings of potentially reversible immunodeficiency such as organ transplant, the lymphomas in CIDs are generally unresponsive because of the inability to remedy the underlying immunologic deficit.

Summary

Lymphomas that develop in the setting of other diseases exhibit a distinct constellation of clinical features. In general, they tend to be of the non-Hodgkin's type and often have a large B-cell, particularly immunoblastic, histology. Difficulty in establishing histopathologic diagnoses may relate to a spectrum of lesions ranging from polyclonal LPD to frankly malignant monoclonal lymphoma. Extranodal, notably CNS, presentations are common. These non-Hodgkin's lymphomas are aggressive and, although responsive to therapy, often lead to death from treatment-related toxicity, uncontrolled lymphoma growth, or progression of the underlying disease. Within the confines of these common features, the LPDs associated with each disease entity have their own peculiar features regarding histopathology, site, and clonality. However, two phenomena dominate the picture of most of these lymphomas. First, all these disease conditions are characterized by some degree of immunodysregulation. Second, a strong association with EBV has been documented in many of these LPDs. A multistep model of tumor progression has been proposed to explain tumorigenesis in the setting of underlying diseases. The observed associations with EBV and immunodeficiency offer likely mechanistic explanations for the progressive steps involved in lymphomagenesis. Finally, while many patients can be successfully treated with conventional chemotherapy, a variety of novel therapies, including biologically based agents, are being tested in an effort to improve response and survival.

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Part V

Supportive Care

48 Supportive Care for Patients with Leukemia: A Historical Perspective

Charles A. Schiffer

Introduction

Once upon a time, the delivery of therapy to patients with acute leukemia was profoundly constrained by problems with venous access, life-threatening infections due to a paucity of effective and nontoxic broad spectrum antibiotics and antifungal agents, hemorrhage due to a lack of readily available high quality platelet transfusions, severe vomiting resulting in weight loss and inanition, and a considerable risk of hepatitis from blood product transfusions. Indeed, the therapeutic nihilism which permeated the medical literature in the late 1960s and early 1970s derived as much from doubts about the ability to keep patients alive following chemotherapy as from skepticism about the effectiveness of the chemotherapy itself. In fact, the current "standard" chemotherapy for acute myeloid leukemia (AML), so-called "3 & 7" (anthracycline and cytarabine), and multi-agent therapy for acute lymphoblastic leukemia (ALL) is essentially identical to what was used and developed in the 1970s.

Forty years later, the situation is profoundly different. As but one example, whereas ~30% of older patients receiving induction therapy for AML experienced early death due to complications of pancytopenia as recently as 20 years ago, a recently published trial in AML patients with a median age of 67 years reported a death rate of only 11% within 30 days of treatment [1]. In this chapter, I will briefly provide my idiosyncratic perspective on the advances in supportive care which contributed to the improved tolerability of induction therapy for acute leukemia.

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Transfusion Medicine

Therapies for acute leukemias became more intensive in the late 1960s creating a demand for more sophisticated supportive care and in particular, a need for a reliable source of high quality platelets for transfusion. Much of the initial clinical investigational work was done in specialized units created within cancer centers that focused on leukemia treatment. Subsequently, as the technology became more standardized and mature, regional blood centers adopted these techniques and high quality red blood cell and platelet transfusion products are now readily available throughout the United States. A large and sophisticated subspecialty termed "Transfusion Medicine" has evolved through the years.

Platelet Transfusion

In the early 1970s, platelets for transfusion were obtained as a by-product of whole blood donation and stored as platelet concentrates at 4 °C. Pools of platelet concentrate were administered and, although these platelets stored at 4 °C were hemostatically effective, posttransfusion increments and platelet survival were reduced [2]. The transition to storage at ambient temperature (22 °C) with gentle agitation was facilitated by advances in the plastics industry [3]. Pliable plastic bags which could tolerate centrifugation at high speeds were developed. These plastics permitted transmission of oxygen, necessary for continued aerobic platelet metabolism, as well as egress of CO_2 which was produced as a consequence of ongoing metabolism by platelets and contaminating white cells. The outward diffusion of CO_2 as well as the development of appropriate citrate anticoagulant buffers allowed maintenance of plasma pH permissive of prolonged platelet storage.

Indeed, systems were developed in which platelets could be stored for 7 days or more at 22 °C (equivalent to or actually longer than they would have survived in circulation) [4] but, because of descriptions of platelet transfusion associated

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bacteremia, storage has now been limited to 5 days [5]. Studies have shown that under proper conditions, there is minimal effect on platelet recovery with storage up to 5 days [6] with posttransfusion increments considerably higher than what was seen when platelets were stored at 4 °C. Bacteremia from "contaminated" platelet products is rare but should be considered in patients who develop unusually severe, febrile reactions while receiving platelet transfusions. In such circumstances, it is appropriate to discontinue the transfusion, and do blood cultures as well as cultures of the platelet bag, usually in association with beginning parenteral antibiotics, particularly in neutropenic patients.

As transfusions became used more widely, the problem of refractoriness to transfusion due to alloimmunization became a major issue. Approximately 40–50% of patients receiving chronic transfusions developed antibody against HLA A or B antigens resulting in impaired platelet recovery and survival and leaving recipients at risk for hemorrhage [7]. A number of investigators demonstrated that satisfactory increments could generally be obtained by transfusion of platelets from donors whose HLA types were identical to or very similar to those of the recipient, and many centers set up large pools of HLA typed donors for use in alloimmunized patients. A number of interesting observations were made, including demonstration of antigenic similarity of different HLA antigens resulting in the ability to selectively "mismatch" for these antigens [8, 9], as well as discrepancies in the expression of particular HLA antigens on the leukocytes and platelets from the same individual, which also permitted successful transfusion of mismatched platelets, thereby increasing donor availability [8, 10].

In parallel with these findings was the development of sophisticated blood cell separators which permitted the collection of leukocytes and the equivalent of many units of platelet concentrates from single donors. A whole series of different technologies were utilized, all capitalizing on disparities in density of different cellular elements and some evolving from technologies used to separate different components of milk products [11, 12]. The apheresis devices focused originally on platelet and granulocyte collection as well as the occasional therapeutic leukapheresis of patients with leukemia and very elevated white blood cell counts. So-called "single donor" platelets are now used widely, although except in alloimmunized patients, they have not been shown to be preferable to pooled platelet concentrates [13, 14]. Perhaps the most common current indications for apheresis are therapeutic plasma exchange, collection of autologous or allogeneic stem cells from peripheral blood to be used in support of high dose therapy, procurement of autologous T cells to be modified ex vivo and used in a variety of "immunotherapy" treatments, and as part of programs of red blood cell exchange for patients with sickle cell disease.

The creation of large registries of HLA type donors was expensive and never became readily available at many centers, particularly as leukemia treatment evolved to the point where most patients were treated in community rather than subspecialty centers. Over the years, a number of assays for platelet antibody testing were developed, many deriving from research focused on the autoantibodies responsible for platelet destruction in patients with immune thrombocytopenic purpura (ITP). Although initially somewhat cumbersome, a number of these assays can now be done rapidly and in a semiautomated fashion, permitting cross matching of donor platelets with patients' sera, conceptually analogous to cross matching for red blood cell transfusion [15, 16]. Most blood centers do cross matching of single donor units which they have been routinely collecting as part of their platelet inventory and this approach has greatly facilitated the management of alloimmunized patients.

Early studies of the histocompatibility antigen system demonstrated that it was not the platelet, but rather the leukocytes which contaminated the platelet transfusion, which were recognized by the host immune system provoking the development of alloantibody. Apheresis technology was further refined to reduce the number of contaminating leukocytes, but the major advance occurred with the development of "inline" filters through which the platelet transfusions were administered and which produced a 3–4 log reduction in the number of mononuclear cells. The Trial to Reduce Alloimmunization to Platelets (TRAP) study demonstrated that alloimmunization rates, as assessed clinically by the measurement of posttransfusion increments and serologically by the measurement of lymphocytotoxic antibodies [17], were substantially reduced in patients who received either pooled platelet concentrates or single donor platelets which had been filtered, compared to the control standard of unfiltered pooled platelet concentrates [13]. There was no advantage of the single donor compared to the filtered platelet concentrates. Because of this critical observation, filtration of blood products, with most blood centers actually filtering the entire red blood cell collection (so-called "pre-storage" filtration), has become routine practice and has markedly decreased the incidence of alloimmunization and simplified the platelet transfusion support of patients with leukemia, aplastic anemia, and recipients of stem cell transplantation. The incidence of febrile transfusion reactions, which are a consequence of infusion of cytokines activated by leukocyte metabolism and death during storage, has also decreased appreciably due to removal of WBC pre-storage [18].

Lastly, there has been a major change in the practice of prophylactic platelet transfusion in the last decade. An early descriptive study from the National Cancer Institute demonstrated an increased incidence of hemorrhage in leukemia patients at platelet counts less than 20,000/μL [19]. Of importance, however, is that the antibiotics available at the time that study was done had inconsistent activity against gram-negative

organisms, the results of chemotherapy were poor resulting in more prolonged periods of aplasia and, perhaps most notably, aspirin was often used as an antipyretic. Indeed, the authors of that paper stated that: "A 'threshold' concept would be useful for the description of a direct causal relationship. For platelet values above the threshold would be adequate to prevent hemorrhage, whereas at levels below this threshold, hemorrhage would occur. This type of relationship *does not exist* between platelet count and hemorrhage" [19]. Nonetheless, in large part based on this publication, it became routine practice for a couple of decades to provide prophylactic platelet transfusion to maintain counts above 20,000/μL.

Subsequent randomized trials demonstrated that utilizing a threshold for prophylactic transfusion of less than 10,000/μL was as safe as the 20,000/μL threshold and resulted in the use of fewer platelet transfusions [20, 21]. Other studies have suggested that lower thresholds are also safe and have emphasized the point that the transfusion "trigger" needs to be individualized, taking into account the presence (or not) of other complicating clinical problems which could increase the risk of hemorrhage in individual patients [22]. Certainly, many clinically stable patients who are not receiving active therapy, including patients with aplastic anemia or myelodysplasia, can remain stable without significant hemorrhage for long periods of time with platelet counts of 5000/μL or less.

Two recent large randomized trials evaluated the safety of a therapeutic transfusion strategy, administering transfusions should signs of hemorrhage develop, compared to the traditional prophylactic approach. Both trials suggested that the 10,000/μL threshold for prophylactic transfusion is still appropriate for patients with hematologic malignancies receiving active treatment, but that there was no difference in the incidence of > grade 2 hemorrhage in patients undergoing autologous stem cell transplantation, perhaps because of the short duration of count suppression following stem cell infusion [23–25]. It is important to note that whatever strategy is used, the incidence of severe and/or fatal bleeding in patients receiving leukemia induction therapy or undergoing stem cell transplantation is remarkably low, a testament to the resilience of the vascular system even at very low platelet counts and even in the setting of other active inflammatory or infectious complications. Guidelines addressing the common clinical issues related to platelet transfusion were published and recently updated by the American Society of Clinical Oncology [26] and by the American Association of Blood Banks [27].

Treatment and Prevention of Infections

Bacterial Infections

There is a well-described relationship between the depth and duration of neutropenia and the likelihood of acquiring infections during and after treatment of acute leukemia [28]. Perhaps the most critical initial treatment principle was the then heretical recommendation that antibiotics be administered empirically to neutropenic patients with fever, irrespective of whether they had localizing signs of infection and *before* confirmation of infection by culture of blood or body fluids. This practice derived from the recognition that patients with severe neutropenia frequently had delayed onset of the characteristic signs of inflammation and frequently presented only with fever or pain in infected areas without obvious signs of infection on examination or by radiographs. In addition, such infections often proceeded very rapidly with the development of sepsis and bacteremic shock.

Empiric treatment was initially directed at gram-negative organisms such as Pseudomonas aeruginosa or Klebsiella, utilizing combination antibiotic therapy which often included nephrotoxic aminoglycosides [29]. Literally dozens of randomized trials comparing either antibiotic combinations or the use of single broad spectrum antibiotics were conducted in the ensuing decades. In general, it was difficult to demonstrate superiority of one regimen compared to others as long as the broad spectrum antibiotics used were suitable for the flora most prevalent in individual institutions. Currently, most institutions use broad spectrum single antibiotics such as cefepime or imipenem or its family members as empiric therapy, including vancomycin if there is clinical suspicion of skin infection. It is routine to counsel patients to present themselves immediately to their physicians or to emergency rooms for prompt empiric treatment of fever in association with severe neutropenia and this practice has substantially decreased the incidence of catastrophic bacteremia amongst neutropenic patients.

In recent years, bacteremia with gram-positive organisms have become more prevalent, perhaps in part related to the ubiquitous use of indwelling central venous catheters, with the gastrointestinal tract as another major source of these organisms [30]. Although infections with commensal bacteria such as Staphylococcus epidermitis are generally of low virulence without subsequent organ damage, they are often partially or completely resistant to even broad spectrum antibiotics and frequently require treatment with vancomycin after they are identified in blood cultures. The spectrum of infecting organisms continues to change and evolve under the pressure of widespread antibiotic use, with infections with vancomycin resistant enterococcus, *Clostridium difficile*, and multidrug resistant Acinetobacter and Klebsiella species as a few recent examples. There are thus still career opportunities in infectious disease focused on myelosuppressed patients.

Attempts to *prevent* infections in neutropenic patients also proceeded in a step-wise fashion throughout the years. Attention to personal hygiene, dental prophylaxis prior to chemotherapy, and careful care of indwelling venous catheters

remain the most important and simplest modalities of infection prevention. Initial attempts at patient isolation in protected environments failed to produce important reductions in serious infections and were very expensive and isolated patients both emotionally and physically to a generally unacceptable degree [31, 32]. Because the gastrointestinal tract was the most common source of gram-negative organisms, early studies attempted GI tract sterilization using broad spectrum oral antibiotic cocktails [33, 34]. These treatments frequently resulted in severe nausea, vomiting (particularly in the pre-antiemetic era), and diarrhea and quickly fell by the wayside. Later experiments attempted to preserve the anaerobic flora of the GI tract because of evidence that the continued presence of these organisms could prevent subsequent colonization by gram-negative bacteria. A series of studies with trimethoprim/sulfamethoxazole also failed to be of significant benefit, however [35, 36]. These approaches were succeeded by a variety of trials utilizing either partially absorbable antibiotics or more broad spectrum antibiotics such as ciprofloxacin; at this time there remains little consensus about the validity and importance of these prophylactic approaches. Nonetheless, because of overall improvement in the quality of antibiotics and greater attention to the need for immediate empiric therapy, death from bacterial infection is now an uncommon problem limiting the administration of initial induction chemotherapy, a dramatic improvement from the earlier days of anti-leukemia chemotherapy.

Fungal Infections

As the death rate from bacterial infections decreased and as patients remained on systemic antibiotics for longer periods of time, infections with yeast and fungal organisms became a major cause of infectious death particularly in patients with very prolonged periods of neutropenia. As with the approach to bacterial infections, guidelines were developed recommending earlier empiric use of antifungal agents in neutropenic patients receiving antibiotics with persistent fever or signs of infection, and in particular sino-pulmonary infections. Empiric therapy was felt to be necessary in this situation because it was very uncommon for blood cultures to be positive with infections with *Aspergillus* sp. or other molds and it was often difficult to biopsy affected tissues because of thrombocytopenia. For decades, amphotericin B was essentially the only available broad spectrum antifungal agent. Although the development of liposomal preparations somewhat ameliorated the side effects [37], this was very difficult therapy associated with fevers, chills, renal insufficiency, hypokalemia, hypomagnesemia, and exacerbation of cytopenias. Therapy often had to be continued for weeks to months, complicating and sometimes precluding the administration of further chemotherapy.

The landscape has changed dramatically in the past 10–15 years. Newer antifungal agents such as voriconazole and caspofungin and their analogues have almost totally supplanted "shake-and-bake" as initial empiric therapy when fungal or yeast infections are suspected [38, 39]. The importance of the avoidance of the debilitating effects of amphotericin B cannot be underestimated and has profoundly simplified and streamlined the care of patients with leukemia. Recent trials have also shown prophylactic benefit from the use of these agents in selected patient populations [40].

Granulocyte Transfusions

The close relationship between the level and duration of granulocytopenia and the incidence of infections prompted interest in the development of blood cell separators to collect granulocytes from normal blood donors for transfusion. This turned out to be easier said than done. Granulocytes have a density similar to lighter red blood cells and it proved to be difficult to efficiently separate the granulocytes from other blood components, even after the availability of apheresis technology which was highly effective at platelet collection and plasma exchange [41].

The initial experience with leukocyte transfusion used leukocytes procured from patients with chronic myelogenous leukemia (CML) from whom very high numbers of cells could be collected by simple centrifugation as well as by apheresis [11, 42]. Prompt clinical responses were seen in some recipients with otherwise refractory infections [11] and in some patients, the infusion of immature myeloid precursors capable of further division provided sustained circulation of granulocytes for days after a single transfusion [43].

After this "proof of principle," which was quite convincing to many of us at that time, attention turned to procurement of granulocyte from normal donors. A number of small randomized trials, which would be considered historically "quaint" by current design and statistical standards, suggested clinical benefit in patients receiving granulocytes obtained from normal donors compared to controls treated with antibiotics alone [44–46]. Nonetheless, granulocyte transfusion never "caught on" and are now used infrequently. There were multiple reasons for the decline in interest in the use of granulocyte transfusions during the last 30 years including:

- Difficulties in obtaining adequate doses of cells for transfusion with average cell yields <10% of what was obtained from CML donors [12]
- Administrative difficulties in obtaining sufficient donors to provide transfusions for many consecutive days
- Failure to recognize the effects of recipient alloimmunization which prevented migration of nonhistocompatible

granulocytes to sites of infection and which produced sometimes severe transfusion reactions [47, 48]
- Overall improvements in supportive care and the decrease in the number of patients with refractory gram-negative bacterial or fungal infections

Of note is that interest in granulocyte transfusion has been rejuvenated somewhat with the demonstration that higher doses can be collected after pretreatment of donors with granulocyte colony stimulating factor thereby substantially increasing the yield and the number of cells transfused [49]. If the incidence of infections with multidrug resistant bacteria increases or if new chemotherapy is developed which is more effective but with the price of longer periods of aplasia, granulocyte transfusion might return to its previous halcyon days.

Antiemetics

The development of potent antiemetics has been of major benefit to leukemia patients receiving induction and post-remission chemotherapy, and the effects of these advances have been somewhat under appreciated. When mixtures of phenothiazines, barbiturates, and metoclopramide represented the standard backbone of antiemetic therapy, induction chemotherapy was accompanied by severe and often protracted nausea and vomiting, depression and altered cognition due to their CNS side effects, and sometimes intractable diarrhea related to the high doses of metoclopramide needed to produce the antiemetic effect. The latter often resulted in perirectal excoriations sometimes resulting in infection, while the repetitive episodes of vomiting resulted in inanition as well as erosive distal esophagitis. The latter often became secondarily infected with candidal organisms resulting in candidemia and not infrequently the development of disseminated disease and hepatosplenic candidiasis with the need for prolonged treatment with amphotericin B. Nutritional problems were particularly devastating for older patients receiving chemotherapy and the presence of hepatosplenic candidiasis frequently delayed and sometimes precluded the administration of further chemotherapy.

With the availability of the serotonin antagonist ondansetron and its offspring, nausea and vomiting became a bad memory of the past and it is now common to see patients eating regular diets without vomiting during their entire period of chemotherapy. Although the increased use of fluconazole as antifungal prophylaxis may have also been contributory, hepatosplenic candidiasis is now extremely rare and I have not seen a case in many years. Because they are generally nutritionally replete, many patients who are not requiring systemic antibiotics can now be discharged after completion of induction or re-induction chemotherapy and followed closely as outpatients. This is a remarkable and underappreciated change and indeed, this single improvement in supportive care makes it more difficult to compare outcomes of more recent trials with those conducted in the pre-antiemetic era.

Hepatitis Testing

A less appreciated advance occurred as a result of the development of sensitive testing for the presence of hepatitis causing viruses transmitted by blood transfusion. So-called "non-A, non-B" hepatitis was a common occurrence in leukemia patients receiving transfusions from sometimes hundreds of different donors during their induction chemotherapy. Signs and symptoms of hepatitis frequently developed towards the end of induction chemotherapy and often resulted in delay of count recovery as well as important delays in the administration subsequent post-remission chemotherapy because of concerns about the administration of potentially hepatotoxic drugs such as cytarabine during periods of active hepatitis [50]. The identification of the hepatitis C virus with the rapid implementation of universal screening of blood has virtually eliminated this as a complication [51]. Of note is that transmission of cytomegalovirus has also markedly decreased by the elimination of white blood cells by filtration of blood and platelet products, decreasing the frequency of new CMV infections in transplant and leukemia patients [52].

Conclusions

It has been interesting to observe the maturation of what was once considered to be "high tech" to the point where these treatments are now routine and sometimes taken for granted by the current generation of clinicians. These improvements in supportive care have allowed the delivery of curative therapy to tens of thousands of leukemia and transplant patients and it is predictable that further fine tuning of these principles will continue to benefit patients in the future.

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Prevention of Infections in Patients with Hematological Malignancies

49

Marcio Nucci and Elias J. Anaissie

Introduction and General Principles: A Risk-Targeted Approach

Infection is a frequent complication and a leading cause of morbidity and mortality in patients with hematological malignancies. Problems associated with the management of infections in these patients include difficulties in early diagnosis because the clinical signs of infection are subtle, the low performance of diagnostic tests, and suboptimal response to treatment because recovery of host defenses is a key factor for resolution of infection. Preventing these infections relies on infection control measures and antimicrobial chemoprophylaxis. While infection control measures are safe (but not always effective), the use of antimicrobial agents for prophylaxis of infection is not devoid of problems. Its wide use may increase the possibility of the development of resistance, select for resistant organisms, and increase toxicity and cost. Therefore, any attempt to administer an antimicrobial agent should be accompanied by a reflection of the potential benefits and risks of prophylaxis.

In general, the higher is the incidence of infection, the more beneficial is likely to be antimicrobial prophylaxis. Likewise, the shorter is the period at risk (and therefore the predicted duration of prophylaxis), the higher is the possibility that prophylaxis will work. However, the prediction of an incidence of infection is not simple, and requires an analysis of various factors including patient's prior exposure to pathogens, underlying disease, previous and current treatment, comorbidities, geographic area, and others. Therefore, three questions are critical in defining the appropriateness of antimicrobial prophylaxis: what is the risk for infection; what are the pathogens that predominate in this setting; and what is the period at risk. In this chapter, we describe various strategies directed at the prevention of infections in patients with hematological malignancies, according to this risk-based strategy.

Table 49.1 provides a risk-targeted approach to prophylaxis of infections in patients with hematological malignancies and Table 49.2 presents the most frequent pathogens responsible for infection according to type of immunodeficiency present.

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Table 49.1 Risk factors for infection in patients with hematological malignancies

Risk factor for infection	Risk category	
	Low	High
General condition including organ function		
Performance status	Good	Poor
Renal failure	No	Yes
Liver failure	No	Yes
Lung disease	No	Yes
Diabetes mellitus	No	Yes
Nutritional status	Normal	Impaired
Iron stores	Normal or decreased	Increased
Age	Younger (<40 years)	Older (>65 years)
Smoking	No	Yes
Underlying disease and its treatment		
Tumor burden	None	Large
Likelihood of obtaining control of the underlying disease[a]	High	Low
Disease-related immunosuppression[b]	Absent	Present
Prior chemotherapy	None or minimal	Extensive
Receipt of purine analogues (fludarabine, cladribine, clofarabine) or monoclonal antibodies (rituximab, alemtuzumab)	No	Yes
Exposure to pathogens		
Prior history of infection[c]	No	Yes
Colonization with pathogens (bacteria, fungi)	No	Yes
Nosocomial exposure to potential pathogens (water and airborne pathogens such as *Legionella*, *Aspergillus* spp. and other molds, resistant bacteria, respiratory viruses)	No	Yes
Community-acquired infections, especially respiratory viruses	No	Yes
History of living or visiting areas of endemic infections	No	Yes
Immunogenetics		
Deficiency of MBL	No	Yes
Polymorphism of TLR	Absent	Present
Duration of neutropenia	Short (<7 days)	Long (>10 days)
Severity of oral and gastrointestinal mucositis	Absent or mild	Severe
Chemotherapy regimen	Less intensive	Intensive
Polymorphisms of genes associated with metabolism of chemotherapeutic agents (pharmacogenetics)	Absent	Present
Renal failure[d]	Absent	Present
T-cell immune reconstitution after HCT	Fast	Delayed
Prior chemotherapy	Minimal	Extensive
CMV serostatus	Negative	Positive
Need for additional chemotherapy to control the underlying disease[e]	No	Yes
In vitro manipulation of stem cells[f]	No	Yes
Graft versus host disease and its treatment (in allogeneic HCT)	No	Yes

MBL mannose-binding lectin, *TLR* toll-like receptors, *HCT* hematopoietic cell transplantation, *CMV* cytomegalovirus

[a]Risk assessment in each underlying disease (e.g., age, initial white blood cell count, cytogenetics, immunophenotype, rapidity of cytoreduction in acute lymphoid leukemia; advanced age, de novo vs. secondary leukemia, prior myelodysplasia, cytogenetics, gene mutation profile in acute myeloid leukemia; mutational status of immunoglobulin Vh gene and chromosomal abnormalities in chronic lymphocytic leukemia)

[b]Most common disease-related immunosuppression include: hypogammaglobulinemia (multiple myeloma, low-grade B-cell non-Hodgkin's lymphoma, chronic lymphocytic leukemia), T-cell mediated immunodeficiency (Hodgkin's lymphoma and certain types of non-Hodgkin's lymphoma) and neutrophil dysfunction (acute myeloid leukemia with myelodysplasia)

[c]Infections with higher risk of recurrence include: mycobacteriosis (tuberculosis and others), aspergillosis, pneumocystosis, cytomegalovirus, Herpes simplex and Varicella-zoster virus, toxoplasmosis and strongyloidiasis

[d]Renal failure increases the risk of severe mucositis in patients with multiple myeloma receiving melphalan-based conditioning regimens

[e]Need for additional chemotherapy in lymphoma and acute myeloid leukemia is usually related to relapse of the underlying disease, whereas in multiple myeloma additional chemotherapy is usually part of the treatment strategy

[f]in vitro manipulation of stem cells decreases the content of CD34+ and T-cells, increasing the duration of neutropenia in the early posttransplant period and delaying T-cell immune reconstitution after transplant

Table 49.2 Pathogens likely to cause infection in patients with hematological malignancies according to the predominant type of immunodeficiency

	Skin and mucous membrane disruption	Hypogammaglobulinemia	T-cell mediated immunodeficiency	Neutropenia and neutrophil dysfunction
Bacteria				
Gram-positive cocci				
Coagulase-negative staphylococci	+++	−	−	++
Staphylococcus aureus	+++	−	−	++
Viridans streptococci	+++	−	−	++
Enterococci	++	−	−	++
Streptococcus pneumoniae	−	+++	−	−
Gram-positive bacilli				
Bacillus spp.	++	−	+	++
Corynebacterium jeikeium	++	−	+	++
Listeria monocytogenes	−	−	+++	−
Gram-negative bacilli				
Enterobacteria[a]	++	−	−	+++
Pseudomonas aeruginosa	++	−	−	+++
Other non-fermentative bacteria[b]	++	−	−	+++
Salmonella spp.	+	+	++	+
Legionella spp.	−	++	++	−
Anaerobes				
Clostridium difficile	++	−	−	++
Clostridium septicum	++	−	−	++
Fungi				
Yeasts				
Candida spp.[c], mucosal disease	+	−	+++	−
Candida spp.[c], invasive disease	++	−	−	+++
Cryptococcus neoformans	−	−	+++	−
Trichosporon spp.	++	−	+	++
Molds				
Aspergillus spp.[d]	−	−	++	+++
Fusarium spp.	−/+	−	++	+++
Zygomycetes	−	−	++	+++
Scedosporium spp.	−	−	++	+++
Agents of phaeohyphomycosis	−	−	+	+
Other				
Pneumocystis jirovecii	−	−	+++	−
Histoplasma capsulatum	−	−	+++	−
Viruses				
Herpes simplex	++	−	+++	++
Varicella-zoster	−	−	+++	−
Cytomegalovirus	−	−	+++	−
Epstein–Barr virus	−	+	+++	−
Respiratory viruses[e]	+	+	++	−
Hepatitis A, B and C	−	+	+	−
Parvovirus	−	++	++	−

(continued)

Table 49.2 (continued)

	Skin and mucous membrane disruption	Hypogammaglobulinemia	T-cell mediated immunodeficiency	Neutropenia and neutrophil dysfunction
Parasites				
Strongyloides stercoralis	–	–	++	–
Toxoplasma gondii	–	–	++	–
Cryptosporidium parvum	–	+	++	–
Mycobacteria				
Mycobacterium tuberculosis	–	–	+++	–
Rapid growing mycobacteria	++	–	+	–
Mycobacterium avium Complex	–	–	+++	–

(−): no; (+): occasional; (++): frequent; (+++): very frequent
[a]Most frequent: *Escherichia coli*, *Klebsiella pneumoniae*, *Enterobacter* spp.
[b]Most frequent: *Acinetobacter* spp., *Stenotrophomonas maltophilia*
[c]Most frequent: *C. albicans*, *C. glabrata*, *C. tropicalis*, *C. parapsilosis*
[d]Most frequent: *A. fumigatus* (~90%), *A. flavus*, *A. terreus*, *A. niger*
[e]Most frequent: Respiratory syncytial virus, Influenza A and B, Parainfluenza 1–3, Adenovirus, rhinovirus, coronavirus, metapneumovirus

Infection Control Measures

Patients and health care workers should be educated about the risk of and methods to prevent acquisition of pathogens. These methods include:

Personal Hygiene

Handwashing

Handwashing remains the simplest and most effective measure to prevent the acquisition of organisms by patients [1]. Patients and health care workers should wash their hands before eating, smoking, or inserting or removing contact lenses, and after using the restroom, blowing their nose, coughing, sneezing, handling dirty items such as garbage, and after touching an animal. In addition, health care workers should also wash their hands between patients. All surfaces should be thoroughly cleaned, including wrists, palms, back of hands, fingers, and under the fingernails, preferably with an alcohol-based hand rub [2]. However, if hands are visibly dirty or soiled with blood or body fluids, soap and water are best for cleaning hands [3]. Additional recommendations include the removal of rings prior to handwashing, keeping nails short and clean, and avoiding the use of artificial nails as they may carry pathogens [4].

Skin and Mucosal Care

The skin flora could potentially be a source of infections. Patients should keep their skin clean with daily baths using an antiseptic solution with special attention to potential portals of infection such as the perineum, and catheter sites.

The oral flora can lead to infection especially in the setting of severe mucositis, after radiotherapy, or in patients with graft vs. host disease (GVHD). Recommendations to maintain a good oral and dental hygiene include: (a) oral rinses 4–6 times a day with sterile water, normal saline, or sodium bicarbonate; (b) tooth brushing at least twice a day with a soft or ultrasoft toothbrush. Swabs are less effective, but should be used if the patient cannot tolerate brushings.

Handling Pets

Pet owners should follow the following recommendations [3, 5]: (a) avoid contact with young animals as pets (higher risk of shedding *Salmonella* spp. and *Campylobacter* spp. because of a higher incidence of diarrhea); (b) obtain veterinarian consultation when a new pet is adopted and yearly thereafter; (c) keep pet's vaccinations current; (d) keep pet's feeding areas clean and its litter box away from kitchen and eating areas; (e) feed pets only with high-quality commercial pet foods, cooked egg, poultry and meat products, and pasteurized dairy products, and avoid access to garbage; (f) supervise pets when they are outdoors to prevent contact with other pet's feces; (g) prevent animals from roaming through tick-infested woods; (h) wash hands after handling pets and avoid contact with pet's feces and bird droppings; (i) avoid contact with animals with diarrhea, dogs exposed to shows or kennels, wild birds (especially pigeons), birds with avian tuberculosis, reptiles (high carriage and shedding of *Salmonella* spp.), and swine (source of *B. bronchiseptica*); (j) keep pets away from face and wounds; (k) trim pet's nail short; (l) notify physician immediately if patient is bitten or injured by a pet; (m) instruct kids not to share kisses with the classroom pet; and (n) when cleaning cages, wear a particulate mask and avoid shaking cages.

Other Personal Hygiene Items Including Food Handling

High-risk patients should follow additional precautions to prevent serious infections as summarized in Table 49.3.

Table 49.3 Instructions to give to patients with hematological malignancies

Apply during periods of severe immunosuppression: Maintain precautions for up to 3 months after last dose of chemotherapy or discontinuation of immunosuppression
Personal hygiene
– Bathe regularly using a mild soap and shampoo and rinse well
– Don't share razors (electric or blade) as they may retain particles of blood
– Wash hands frequently, preferably with liquid soap before eating and after contact with contaminated materials. If not washed, keep hands away from eyes nose and mouth
– Maintain good dental hygiene, by brushing teeth with soft bristle toothbrush, after meals and floss daily. Do not share toothbrushes and change toothbrush every 3 months
– Use disposable vaginal douches, and when menstruating, avoid tampons change sanitary napkins frequently
– Use sitz baths or soothing lotion for irritations of the rectum or vagina
– Prevent skin dryness (use moisturizing creams)
– Keep nails short and clean and avoid nail clippers used by others
– Clip toenails straight across to prevent them from becoming ingrown
– Try to avoid trauma to and irritation to the nails
– Wear cotton gloves for chores that don't involve water and rubber gloves for chores involving water
– Avoid unprotected sexual exposure (HIV, *Human papillomavirus*, *Herpes simplex*, *Hepatitis B*)
Environment
– Discourage visits by individuals with respiratory infections
– Avoid crowded places
– Don't share towels with others
– Keep house and rooms well ventilated and change air filters regularly
– Encourage household members to get influenza vaccine
– Avoid swimming (particularly in stagnant water)
– Ask your doctor for preventive measures before travel
– Avoid exploring caves, cleaning chicken coops (histoplasmosis)
Other patients
– Avoid close contact with infected patients (tuberculosis, herpes zoster, herpes simplex, other)
Medication/vaccination
– Before traveling, consult your physician and take all medications
– Have vaccines according to recommendation of your clinician
Food/water
Precautions for food handling
– Cook food thoroughly, wash fruits/vegetables before eating
– Wash dishes and silverware in hot soapy water and dry then very well
– Keep uncooked meats separate from vegetables, fruits and wash hands, knives, and cutting boards after handling uncooked foods and clean kitchen surfaces that have come in contact with raw meat
– Avoid using tap water for drinking or making juices, other food items
– Refrain from skinning animals or cleaning seafood
– Use plastic bags in all trash cans for proper disposal
– At the supermarket, pick up perishables last and take them home promptly
– Defrost meat, turkey, chicken in the refrigerator
– Wash the meat before cooking
– Cook thoroughly eggs and meat (use thermometers)
– Clean your refrigerator regularly discarding food of >3–4 days age, especially salad dressings, sauces, milk and egg products, condiments, processed meats, bacon
– Never use canned foods if the can is swollen dented, or rusted
– Toss out any cheese or food that's moldy. Cut up fresh cheeses into small portions and store separately in the freezer, taking out only what can be used up quickly
– Keep cold foods cold (<40 °F) and hot foods hot (>140 °F)
– When preparing foods, the hands should be kept away from the hair, mouth and nose. If possible, rings and jewelry should be removed, because they may harbor germs. Try to limit touching food with the hands at all; use tongs or a fork if possible. -after cutting up raw meats, soak the cutting board and all utensils for 30–40 min in solution of one part bleach and eight or nine parts water -one ounce of bleach to a cup of water. All foods that are not going to be cooked should be prepared first; only after those are out of the way, can any raw meat and poultry be prepared
– Wash all fruits and vegetables well
– Keep food preparation surfaces clean, and use a good dishwashing detergent on the work surface often, especially while handling raw meat, chicken, or fish
– Never let cats or other animals up on the work surface
– Do not prepares food if you have diarrhea or vomiting, or have an open infected sore
– Put leftover foods into the fridge right away and divide large leftovers into individual containers (to avoid repeated warming)
Food restrictions
– Raw eggs (sometimes used in restaurant-prepared Caesar salad dressing or homemade mayonnaise, eggnog)
– Dried, uncooked or undercooked meats, seafood and poultry (to include medium or rare steaks, game, pickled fish or oysters), or food from delis such as cold cuts, hot dogs, tofu, sausage, bacon, cold smoked fish, and lox
– Unpasteurized commercial fruit and vegetable juices
– Unpasteurized milk or cheese products
– Soft and aged cheeses such as feta, brie, camembert, blue-veined, −Mexican-style cheese, refrigerated cheese-based salad dressings (e.g., blue cheese). *Cream cheese, cottage cheese or yogurt (provided they do not contain Lactobacillus* spp.*) are ok to eat*
– Unwashed raw vegetables and fruits end those with visible mold
– Unpasteurized honey or beer or raw, uncooked brewer's yeast
– All miso products (e.g., miso soup); tempe (tempeh); mate'tea
– All moldy and outdated food products
– Herbal preparations and nutrient supplements

Environmental Precautions

Hospital Environment

Air Precautions

Air quality is important to prevent infections in high-risk patients by airborne organisms such as molds (*Aspergillus* spp. or other filamentous fungi), *Legionella* spp. and *Mycobacterium tuberculosis.* Patients at very high risk for invasive aspergillosis (IA) should be placed in sealed rooms with HEPA filters (central or point-in-use) and positive pressure. Air flow should be direct (air intake at one side of the room and air exhaust at the opposite side), and the system should be able to make ≥12 air exchanges per hour [6]. This group is represented mostly by patients receiving induction chemotherapy for acute myeloid leukemia (AML) and in the pre-engraftment period of myeloablative allogeneic hematopoietic cell transplantation (HCT).

The conidia levels in outdoor air vary widely, from 1–5 cfu/m^3 [7] to 2400 in winter and fall in certain areas [8]. The safe concentration of airborne fungi is not established and probably depends on the patient's immune status. The efficacy of HEPA filters in preventing the entry of contaminated outside air into the hospital was confirmed after the demolition of a building. Despite the increase in the number of conidia of filamentous fungi, no conidia were found in most HEPA filter-equipped areas [9]. Because construction and renovation may increase the concentration of airborne fungi, guidelines have been developed when such activities are taking place close to areas were high-risk patients are cared for [10].

Portable HEPA filters decrease the concentration of airborne fungal spores [11] and their use has successfully prevented the occurrence of fungal infections during building construction [12]. However, it is generally agreed that they are less efficient than central or point-in-use HEPA filters [3].

Airborne fungi have been shown to secondarily aerosolize from a water source [13]. Therefore, preventive measures to limit exposure to water can decrease the airborne concentration of fungal pathogens (see below).

Diet

Although no data exist to support a role for sterile or low-level microbial-content (<1000 CFU/mL of nonpathogenic organisms) diets for patients with hematological malignancy, this practice is generally recommended [3]. A randomized study compared cooked and uncooked diet for patients undergoing induction remission for AML. There were no differences in the rates of episodes of major infection and death [14]. A Cochrane review published in 2016 found only three studies comparing cooked and uncooked food. Since pooling of results was not possible, and serious methodologic limitations were found, the authors could not provide solid recommendations for clinical practice [15].

Water

The hospital water system can be a reservoir for *Legionella* spp. [16], bacteria [17–19], and the opportunistic molds, especially *Aspergillus* spp. [13, 20–22], *Fusarium* spp. [23, 24], and *Exophiala jeanselmei* [25]. Potential modes of acquisition of infection include contamination of intravenous solutions, direct contact with skin breakdowns, and aerosolization of fungal spores. Measures to prevent the occurrence of infection depend on the mode of acquisition. It is generally recommended that patients at risk for such infections should avoid direct exposure to contaminated water. In addition, specific measures have been tested, including the use of point-in-use water filters for *Legionella* spp. [26] and cleaning water-related structures to prevent aerosolization of fungi [27].

Health Care Workers (HCW)

Infections can be transmitted from the HCW to the patient. The risk of transmission is high for Varicella zoster (VZV), viral conjunctivitis, measles, and tuberculosis, and intermediate for influenza, mumps, Parvovirus B19, pertussis, respiratory syncytial virus (RSV), rotavirus, and rubella. Therefore, HCW with any of the abovementioned infections or with HSV lesions in lips or fingers should not be in contact with patients [3].

HCW who care for patients with hematological cancer should be immunized against rubella, measles, mumps, influenza, and chickenpox, in addition to the already recommended tetanus and hepatitis B immunization [3].

Household Exposure

The recommendations for immunization and precautions that apply to the HCW also apply to close contacts of patients with hematological cancer [3]. Immunization against hepatitis A and B is highly recommended for sexual contacts of patients. In addition, immunization against hepatitis A should be considered for all households of patients with chronic liver disease or living in endemic areas. Oral polio vaccine is contraindicated for all households of patients with hematological cancer since live polioviruses can be transmitted to and cause disease in immunocompromised patients, especially during the first month after vaccination [28]. Patients with hematological cancer should also avoid exposure to individuals with vesicular rash secondary to chickenpox immunization to prevent VZV disease [3].

Sexual partners: Sexually active patients should avoid unprotected sex during the periods of significant immunosuppression to reduce the risk of exposure to CMV, HSV, HIV, HPV, HBV, HBC, and other sexually transmitted infections [3].

Invasive Procedures

Procedures that break the integrity of natural barriers such as skin and mucosa should be avoided when possible. Fixed orthodontic appliances and space maintainers should not be worn during any period of neutropenia to avoid oral trauma and infection. Enemas, suppositories, rectal temperature check, or/and rectal examination are contraindicated. Necessary dental procedures should be performed prior to chemotherapy to allow proper healing before neutropenia and mucositis develop [29]. Bone marrow biopsies should be done aseptically to avoid cellulitis and osteomyelitis.

Recommendations for the insertion of indwelling devices include careful cleaning and sterilization of instruments and devices (particularly reusable ones) and guidelines for the prevention of intravascular device-related infections [30]. However, solid evidence to support some of the guidelines for the prevention of intravascular device-related infections is lacking.

Antimicrobial Prophylaxis

Antimicrobial prophylaxis may be primary, when prevention targets an individual that has not been infected in the past, and secondary, when prevention is used to avoid recurrence of infection in an individual who has been previously infected.

Antibacterial Prophylaxis

Bacterial infections occur frequently in two settings: neutropenia and hypogammaglobulinemia. As shown in Table 49.2, common bacterial infections in patients with neutropenia include staphylococci, enterococci, and viridans streptococci among the Gram-positive bacteria, and enterobacteria and non-fermentative bacteria (especially *Pseudomonas aeruginosa*, *Acinetobacter* spp., and *Stenotrophomonas* spp. among the Gram-negative bacteria).

Because Gram-negative bacteremia may be associated with high mortality rates, strategies of antibacterial prophylaxis during neutropenia have been focused mostly to prevent the occurrence of Gram-negative bacteremia, and the quinolones have been extensively studied. A meta-analysis pooling data from 95 trials showed that quinolones reduced the incidence of fever, documented infections, and mortality associated with infection [31]. A major concern is the development of resistance. Another meta-analysis examined the effect of quinolone prophylaxis on microbial resistance. There was no difference in the incidence of colonization by resistant organisms, or in the rates of infection caused by resistant pathogens [32]. These data, however, must be interpreted with caution, because rates of resistance are very different among different institutions, cities, and countries. As a general rule, once the clinician decides to give prophylaxis with a quinolone for neutropenic patients, a careful attention to the development of resistance is advised.

Another concern when using quinolone prophylaxis is the increase in the incidence of infections caused by Gram-positive organism, notably viridans streptococci [33, 34]. A great concern related to such infections is that they may occasionally evolve to shock and respiratory failure [35]. Although most of such infections may be prevented by penicillin or macrolides [36], some strains are resistant to these agents [37]. The use of glycopeptides is not generally recommended for prophylaxis [3]. Table 49.4 shows the usual doses of quinolones in the prophylaxis of bacterial infections in neutropenic patients.

Hypogammaglobulinemia is frequent in chronic lymphocytic leukemia, multiple myeloma, and in allogeneic HCT

Table 49.4 Dosage-schedule of antimicrobial agents used in the prophylaxis of infection in patients with hematological malignancies

Disease	Prophylaxis
Bacterial infections	
Neutropenic	Quinolone[a]
Non neutropenic	TMP-SMX—800 mg/160 mg PO daily Or daily quinolone
C. difficile diarrhea	Consider metronidazole prophylaxis (500 mg PO TID) if prior history of CDAD
Tuberculosis	Isoniazid—300 mg PO daily
Fungal infections	
Invasive candidiasis	Fluconazole—200–400 mg PO daily
Invasive aspergillosis	Posaconazole—200 mg TID for oral solution or 300 mg BID on day 1 followed by 300 mg once daily on day 2 and thereafter for tablet
Oral and/or esophageal candidiasis	Clotrimazole troches (10 mg, ×5/day) or fluconazole—100–200 mg PO daily
Pneumocystis jirovecii pneumonia	TMP-SMX—800 mg/160 mg PO daily or ×2/week, pentamidine—300 mg aerosol monthly, dapsone—100 mg PO daily, atovaquone 1500 mg PO daily
Viral infections	
Herpes simplex	Acyclovir—200–400 mg PO BID or TID, valacyclovir—500 mg PO TID or famciclovir—500 mg PO TID
Herpes zoster	Acyclovir—400 mg PO BID or TID, valacyclovir—500 mg PO TID or famciclovir—500 mg PO TID
Cytomegalovirus	Ganciclovir—5 mg/kg IV BID or valganciclovir—900 mg/d PO or foscarnet—60 mg/kg IV BID
Influenza virus	Oseltamivir—75 mg PO daily for the duration of the influenza season. Zanamivir is more appropriate in the presence of viral resistance

TMP-SMX trimethoprim-sulfamethoxazole, *PO* per os, *TID* three times a day, *QID* four times a day, *BID* twice a day

[a]Includes ciprofloxacin—500 mg PO BID, levofloxacin—500 mg PO daily, moxifloxacin—400 mg PO daily, others

recipients who develop GVHD. These patients are at greater risk of developing bacterial infections, particularly by encapsulated bacteria. Intravenous immunoglobulin (400 mg/kg) every 4 weeks may be effective for the prevention of bacterial infections, and this recommendation is supported by randomized controlled studies [38–40]. However, since its use is costly, intravenous immunoglobulin should be reserved to a selected population of patients with repeated episodes of severe infections. A meta-analysis of nine studies comparing intravenous immunoglobulin with a control group in patients with chronic lymphocytic leukemia or multiple myeloma did not show any survival benefit of immunoglobulin prophylaxis. However, a reduction in the incidence of major infections and of clinically documented infections was observed. The authors concluded that intravenous immunoglobulin should not be recommended routinely [41]. A cheaper alternative to immunoglobulin is to give quinolone prophylaxis with levofloxacin (500 mg/day), moxifloxacin (400 mg/day), or sulfamethoxazole-trimethoprim (TMP-SMX) (Table 49.4) [42].

Antifungal Prophylaxis

Primary prophylaxis against invasive candidiasis is not indicated in all neutropenic patients. In allogeneic HCT recipients, two randomized clinical trials (RCTs) showed that fluconazole reduced the frequency of superficial and systemic candidiasis, as well as infection-related mortality [43, 44]. In one of these trials, fluconazole was given until day +75 posttransplant, and a post hoc analysis of the trial has shown that fluconazole was associated with prolonged protection against invasive candidiasis, even beyond the period of prophylaxis [45].

The benefit of prophylaxis against invasive candidiasis was not as apparent in other settings, such as in patients with acute leukemia [46]. However, the ineffectiveness of fluconazole in non-HSCT neutropenic patients is probably related to the heterogeneity of the populations of neutropenic patients studied (with different incidences of invasive candidiasis) rather than an absence of efficacy. In general, the higher is the risk for the patient to develop severe mucositis during neutropenia, the higher is the risk for invasive candidiasis.

Fluconazole is the drug of choice, usually at a dose of 400 mg daily. Fluconazole is not effective in preventing infection caused by all *Candida* species. *Candida krusei* is intrinsically resistant to fluconazole, and *Candida glabrata* exhibits minimal inhibitory concentrations (MIC) higher than other species. As a consequence, fluconazole is not recommended for the prevention of infection due to these two species.

Other than fluconazole, itraconazole oral solution (but not capsules) [47], voriconazole [48], posaconazole [49], and micafungin [50] effectively prevent the occurrence of invasive candidiasis during neutropenia.

Invasive aspergillosis usually occurs in the context of prolonged (>15 days) and profound (<100/mm^3) neutropenia in patients receiving induction therapy for AML or myelodysplasia (MDS), or after myeloablative conditioning regimen for allogeneic HCT [51]. In addition, HCT recipients with GVHD are at high risk for IA. In these patients, severe T-cell mediated immunodeficiency rather than profound and prolonged neutropenia is the main risk factor [52]. More recently, cases of IA have been diagnosed in patients with other hematological malignancies, including patients with chronic lymphocytic leukemia receiving treatment with alemtuzumab, and patients with multiple myeloma [53–56].

In the setting of AML/MDS, posaconazole (200 mg 3×/day) was superior to fluconazole or itraconazole oral solution in a large randomized controlled trial, and is considered the drug of choice for anti-*Aspergillus* prophylaxis [49]. By contrast, a reduction in the incidence of IA was not observed in trials comparing itraconazole with fluconazole, and itraconazole was associated with more adverse events [47, 57]. A recent meta-analysis of itraconazole trials suggest that there is a reduction in *Aspergillus* infections but only if a certain threshold of bioavailable dosing is used [58]. Its ability to prevent invasive fungal diseases (IFD) has been associated with trough itraconazole concentrations >500 ng/mL, best achieved with the IV formulation (followed by the oral solution if the gastrointestinal function is intact). The oral capsule formulation suffers from erratic bioavailability and is best avoided.

In allogeneic HCT recipients, itraconazole oral solution resulted in a reduction in the frequency of IA in 2 trials, but about 25% of patients discontinued itraconazole because of gastrointestinal side effects [59, 60]. In these trials, prophylaxis was used both in the early pre-engraftment and in the post-engraftment period. Another randomized clinical trial compared posaconazole to fluconazole in allogeneic HCT recipients who developed GVHD. Although the primary endpoint (incidence of IFD from randomization to day 112 of prophylaxis) was not achieved, posaconazole significantly reduced the incidence of IA [61]. Micafungin given during the pre-engraftment period was associated with a trend suggesting ability to prevent aspergillosis. In this trial the incidence of IA was 0.2% among 425 patients receiving micafungin and 1.5% among 457 patients receiving fluconazole ($p = 0.07$) [50].

Two randomized clinical trials evaluated the efficacy of voriconazole as prophylaxis in allogeneic HCT recipients. In the first study, patients received voriconazole or fluconazole from day zero until day +100 (or beyond, in the presence of GVHD) posttransplant. For the primary endpoint (fungal-free survival at 180 days), no differences were observed in the two arms (75% fluconazole vs. 78% voriconazole, $p = 0.49$). There was a trend for a lower incidence of IA in

voriconazole recipients ($p = 0.09$) [48]. The other study compared voriconazole and itraconazole, given for the same period as the previous study [62]. The incidence of IFD (including IA) was similar in the two arms, but tolerability was better with voriconazole.

Taken together, it seems that mold-active azoles indeed reduce the incidence of IA. These findings, however, should be balanced against our significantly improved ability for the early detection of fungal infections and the potential undesirable consequences including toxicities, drug–drug interactions, costs, and emergence of resistance [63]. The application of serial serum galactomannan monitoring has enabled us to make the diagnosis of IA much earlier, with a significant impact in reducing mortality [56]. Indeed, in the trial comparing voriconazole and fluconazole, screening with twice-weekly serum galactomannan was part of the protocol in the two arms, and appropriate antifungal therapy was started based on positive galactomannan tests. The absence of a significant difference in the incidence of IA suggests that giving an anti-mold agent as prophylaxis or giving fluconazole plus serial monitoring with serum galactomannan results in similar outcomes.

Therefore, several factors should be taken into consideration in determining if prophylaxis is appropriate at a specific treatment center, for a given patient or patient population to target a specific infection or if prophylaxis should be withheld and a diagnostic-based preemptive strategy used instead. In general, the higher the risk, the more likely anti-mold prophylaxis should be given. Therefore, risk assessment (Table 49.1) should be performed in order to decide the best strategy. Patients at high risk to develop IFD should receive anti-mold prophylaxis, while low-risk patients can be managed with fluconazole plus active monitoring with serial (3×/week) serum galactomannan and CT scans, provided that these tools are available in the hospital [64]. Clinicians should keep in mind that this risk assessment is dynamic. For example, a patient with AML who was considered at low risk on admission but presents residual blast cells on day 15 of admission should be reclassified to a higher risk and the prophylactic regimen should be changed accordingly [64].

Secondary prophylaxis is indicated for patients who developed an invasive mold infection and will receive treatment for the underlying malignancy that results in immunosuppression, particularly neutropenia and/or T-cell immunodeficiency [65]. Options for secondary prophylaxis include amphotericin B and its lipid formulations, caspofungin, itraconazole, voriconazole and lipid amphotericin B followed by voriconazole [66–70]. In addition to secondary chemoprophylaxis, strategies to abbreviate the duration of neutropenia, such as the use of reduced-intensity conditioning regimens and peripheral blood stem cells, and the use of granulocyte transfusions may be employed [71, 72]. The antifungal agents and doses given as prophylaxis are summarized in Table 49.4.

Antiviral Prophylaxis

Most viral infections that complicate the course of chemotherapy in patients with hematological malignancies represent reactivation of latent infections, while a minority are due to exogenous acquisition (such as respiratory viruses).

Cytomegalovirus (CMV)

Until the early 1990s, CMV seropositive allogeneic HCT recipients had a 70–80% risk of viral reactivation, and one-third of these patients developed CMV disease (mainly pneumonia) [73] with a high fatality rate [74]. The application of preemptive therapy guided by serial monitoring with CMV antigenemia has markedly reduced the incidence of patients who develop overt manifestations and/or die of CMV pneumonia [75]. More recently, quantitative PCR for the detection of CMV DNA and CMV RNA have been introduced as alternatives for the antigenemia [76, 77]. Since these techniques are more sensitive than antigenemia, a threshold for starting preemptive therapy should be established for every group of patients; in other words, a specific number of copies of CMV DNA above which triggers the institution of preemptive therapy in allogeneic HCT may not be the same for patients with less severe immunodeficiency. Indeed, the application of these sensitive biomarkers has revealed that hosts not thought to be at risk for CMV reactivation may indeed have positive CMV PCR quite frequently [78]. Outside the setting of allogeneic HCT, patients at higher risk to develop CMV reactivation include patients with chronic lymphocytic leukemia (CLL) receiving fludarabine or (especially) alemtuzumab [79], patients with multiple myeloma receiving highly intensive therapies [80], and autologous HCT recipients treated previously with rituximab [81].

Two strategies were reported effective for the prophylaxis of CMV disease in allogeneic HCT recipients: universal prophylaxis and preemptive therapy. Universal prophylaxis is not usually given because this strategy may lead to a significant increase in the incidence of bacterial and fungal superinfections associated with ganciclovir-induced neutropenia and immunosuppression [82], and the occurrence of late CMV disease [74]. Ganciclovir, administered intravenously, is the drug most often used for preemptive therapy. The usual duration of therapy is 2 weeks, provided antigenemia (or PCR) becomes promptly negative. Otherwise, a prolonged course of ganciclovir or maintenance therapy is indicated. Alternatives to ganciclovir include foscarnet and oral valganciclovir [3]. Investigational agents include brincidofovir [83], letermovir [84], and maribavir [85].

Herpes Simplex Virus (HSV)

Reactivation of HSV is frequent in patients with hematological malignancies, especially after induction chemotherapy for acute leukemia, and following conditioning

regimens for HCT, and manifests as oral lesions indistinguishable from chemotherapy-induced mucositis [86]. Less frequent manifestations include genital ulcers, esophagitis, hepatitis, and pneumonia. Antiviral prophylaxis against HSV is administered if the patient is seropositive for HSV or conveys a history of recurrent fever blisters, cold sores, or other indications of recurrent HSV infections, particularly if the CD4 counts are low (<50/mm^3). The drug of choice is acyclovir, and should be given prior to or at the time of cytotoxic or myeloablative chemotherapy and continued until bone marrow recovery and/or resolution of mucositis [86]. Alternatives to acyclovir are valacyclovir and famciclovir (Table 49.4).

Varicella-Zoster Virus (VZV)

Patients at highest risk for VZV reactivation are those with severe lymphopenia and/or CD4 cytopenia such as patients with lymphoma, leukemia (mainly CLL), heavily treated myeloma patients, HCT recipients and patients receiving fludarabine or alemtuzumab. Without acyclovir prophylaxis, reactivation of VZV is common and can be complicated with severe post-herpetic neuralgia. Visceral dissemination (pneumonitis, meningoencephalitis, and hepatitis) may rarely occur in severely immunocompromised patients [87].

Patients at high risk should avoid contact with persons with VZV disease, as well as vaccine recipients who develop a rash after vaccination. In addition, contact and airborne precautions are recommended if an immunocompromised patient develops VZV disease, in order to decrease the risk of transmission to other patients and to HCW [3].

High-risk patients with a history of recent contact with any person with VZV disease should receive varicella-zoster immunoglobulin (VZIG) or, as an alternative, acyclovir or valacyclovir [88]. Acyclovir is indicated as prophylaxis against VZV reactivation in allogeneic HCT recipients, usually given for 1 year [89]. In addition, patients with multiple myeloma receiving regimens containing bortezomib should receive prophylaxis because of the high risk of VZV reactivation [80]. The use of VZV prophylaxis in other settings is more debatable and should be reserved for severely immunosuppressed patients, especially if they develop herpes zoster.

Epstein Barr Virus (EBV)

Patients with EBV disease may present with fever and mononucleosis syndrome. In addition, HCT recipients may present with posttransplant lymphoproliferative disease (PTLD). Patients at high risk for PTLD include recipients of matched unrelated, mismatched, or T-cell depleted transplants, recipients of high dose antithymocyte globulin or anti-T-cell monoclonal antibodies, patients with acute and chronic GVHD, and those receiving radiation as part of the conditioning regimen [90].

High-risk patients who are EBV seronegative should be advised to avoid close contact with EBV seropositive individuals. Increases in EBV viral load following PPSCT/BMT are common, and are highest in patients at risk for PTLD. The best strategy to prevent PLTD is to serial monitor high-risk patients with serum quantitative PCR technique and giving rituximab preemptively for patients who present EBV replication [91].

Hepatitis B Virus (HBV) and Hepatitis C Virus (HCV)

Patients with hematological malignancies undergoing cytotoxic chemotherapy while infected with HBV have a higher risk for severe liver dysfunction [92]. During therapy-induced aplasia, the possibility of viral replication increases dramatically resulting in acute HBV infection that may be mild, asymptomatic, or chronically progressive leading to fulminant hepatitis. Fulminant hepatitis usually coincides with discontinuation of immunosuppression [93]. Risk factors for reactivation include male gender, younger age, a diagnosis of lymphoma, and positive HBV e antigen [94]. In HCT recipients, the risk of reactivation is as high as 50% [95]. Although any chemotherapy regimen may result in HBV reactivation, the risk is particularly higher after exposure to corticosteroids, rituximab, and alemtuzumab [96, 97].

Patients with circulating HBV DNA should receive preemptive therapy with lamivudine (100 mg/day). This regimen is effective and relatively nontoxic. However, prolonged exposure to lamivudine may result in the development of resistance. The optimal duration of preemptive therapy is not established, but is usually recommended to be at least 6 months after discontinuation of chemotherapy, to avoid viral reactivation and the development of hepatitis [95, 98].

Patients infected with HCV may receive chemotherapy or HCT without major complications except for a higher risk for sinusoidal obstruction syndrome; the risk for such patients is the development of late cirrhosis, several years after HCT [99]. Patients with HCV should be assessed for the evidence of chronic liver disease. Patients with cirrhosis who are selected for receipt of HCT should not receive conventional conditioning regimens. Although oral ribavirin may clear HCV viremia, its routine use as prophylaxis is not recommended.

Respiratory Viruses

The respiratory viruses Adenovirus, Influenzae, Parainfluenza, Respiratory Syncytial Virus (RSV), Rhinovirus, Coronavirus, and Metapneumovirus may cause infections in patients with hematological malignancies. Most of these infections appear to be self-limited, although progression to severe lower respiratory infection may occur [100–103]. The main strategy for prophylaxis of infections by respiratory viruses is to prevent

exposure of patients with hematological malignancies to individuals with symptoms of respiratory infections.

Vaccination of household contacts and HCWs for Influenza is recommended during each Influenza season [3]. In addition, patients receiving chemotherapy should also receive the vaccine, considering that they may be able to respond vaccination and the intervention is safe [104]. However, considering that the response to vaccination may be suboptimal, chemoprophylaxis with neuraminidase inhibitors during a community outbreak has been recommended [3].

Regarding RSV, parainfluenza virus, and adenovirus, while highly immunosuppressive patients may be at risk for severe pneumonia, no formal prophylaxis is available and approved. Therefore prevention of severe disease is best approached by early diagnosis and therapy.

Other Pathogens

Mycobacteria

The incidence of tuberculosis in patients with hematological malignancies is low, even in highly endemic regions. In a study from Spain, the incidence of tuberculosis was significantly higher than the general population among allogeneic but not autologous HCT recipients [105]. In another study, 917 patients with hematological malignancies from Brazil were retrospectively reviewed for a diagnosis of tuberculosis. The prevalence was 2.6% only; risk factors were an underlying disease associated with significant impairment in CMI (e.g., receipt of fludarabine and corticosteroids) and malnutrition [106]. The problem is that most patients who develop tuberculosis have not had clearly identified risk factors.

Patients should avoid contact with persons with active tuberculosis, as well as environments that may potentially have patients with tuberculosis, such as health care facilities and shelters for the homeless. There are no studies testing antimicrobial prophylaxis in high-risk patients. Recently published guidelines for infection prophylaxis in HCT recipients recommend the use of isoniazid (5–10 mg/kg, maximum, 300 mg/day) with pyridoxine 25 mg daily for >9 months and until immunosuppression dosages are substantially reduced in patients with past history of tuberculosis or exposure to someone with active tuberculosis, patients with positive tuberculin test or interferon-gamma release assays without a history of BCG vaccination [3].

Pneumocystis jirovecii

Reactivation of latent infection is the most common mechanism of pneumonia by *Pneumocystis jirovecii* among immunocompromised patients. Patients at high risk for *Pneumocystis jirovecii* pneumonia (PJP) are those with chronic T-cell immunodeficiency, particularly: children with acute lymphoid leukemia (ALL), HCT recipients, and patients receiving purine analogues, monoclonal antibodies, or corticosteroids for long periods [107, 108].

The most effective drug for prophylaxis is TMP-SMX. Accepted dosages include one double-strength tablet (trimethoprim 160 mg + sulfamethoxazole 800 mg) bid 2 days a week, one double-strength tablet (daily or 3 times a week), and 1 single-strength tablet (trimethoprim 80 mg + sulfamethoxazole 400 mg) daily. The time of initiation and the duration of prophylaxis should be individualized according to the underlying disease and type of treatment. For example, in ALL patients, prophylaxis is usually started at the end of the induction period and discontinued 3 months after completion of maintenance therapy; in HCT recipients it should be started after engraftment and continued as long as immunosuppressive therapy is ongoing, extensive GVHD is present and CD4 count is <200 cells/mm^3. Alternative agents include: aerosolized pentamidine (given with Respirgard II nebulizer 300 mg every month after an initial loading dose given every other week), atovaquone suspension (1500 mg/day), and dapsone (50 mg bid or 100 mg/day) [3, 109].

Toxoplasmosis

Seropositive patients are at risk of reactivation of toxoplasmosis following HCT. When the recipient and donor are seronegative, special precautions should be taken to avoid primary infection. Those precautions include eating only well-cooked meats (>66°C), well-washed vegetables, cooked eggs, pasteurized milk, sterile water, handwashing after outdoor activities or after handling raw meat or vegetables, using gloves for contact with soil or gardening, avoiding contact with cat litter, and having someone change litter box daily and soak it in boiling water for 5 min.

Reactivation of toxoplasmosis is highest among recipients of T-cell depleted allogeneic PSCT/BMT (5–15%) and is otherwise rare among other allogeneic recipients (<1%). The potential toxicities of effective agents preclude routine prophylaxis against toxoplasmosis. However, preemptive therapy of high-risk patients (positive serology prior to transplantation, T-cell depleted allogeneic transplants) with PCR-based tests is recommended. Primary prophylaxis may be considered in patients with history of ocular toxoplasmosis. Effective prophylaxis includes TMP-SMX, one double-strength tablet daily or 3 times a week, or 1 single-strength tablet daily, Clindamycin 300–450 mg thrice daily plus pyrimethamine 25–75 mg/day plus leucovorin 10–25 mg, pyrimethamine-sulfadoxine (Fansidar) 1 tablet (25 mg pyrimethamine/500 mg sulfadoxine)/20 kg weight on day 1 with folinic acid, 50 mg/20 kg on day 2, then daily following engraftment, atovaquone (750–1500 mg/day), and dapsone 50 mg/day plus pyrimethamine 50 mg/week plus folinic acid 25 mg/week. Fansidar is associated with significant toxicities [110].

Other Parasites

Strongyloides stercoralis may cause a fatal disseminated syndrome with intestinal larval invasion and bacterial superinfection. Patients at high risk are those with T-cell immunodeficiency [111]. Patients at risk should avoid contact with outhouses and cutaneous exposure to soil or other surfaces that might be contaminated with human feces. In addition, patients with unexplained eosinophilia, or those who live in, have resided, or traveled to endemic areas should be screened with either stool examinations (≥3 stool examinations), or an enzyme-linked immunosorbent assay (ELISA) [112]. Patients whose screening is positive should receive empiric treatment with ivermectin (200 μg/kg/day for 2 days, repeat after 2 weeks) [113].

Immune Reconstitution

Passive Immunization (IV Immunoglobulin, IVIG)

Intravenous immunoglobulins may benefit patients with CLL, non-Hodgkin lymphoma and myeloma who have severe hypogammaglobulinemia (serum IgG levels <500 mg/dL) and recurrent and/or severe infections despite appropriate antimicrobial prophylaxis and immunizations [38–40]. Doses of IVIG of 250 mg/kg every 4 weeks were shown to be as effective as 500 mg/kg every 4 weeks. However, the role of IVIG in the prevention of infections among patients with hematological malignancies is not clear and is unlikely to be superior to that of antibiotic prophylaxis (see Antibacterial Prophylaxis above). Therefore, IVIG should probably be given to patients with hypogammaglobulinemia and recurrent bacterial infections despite prophylactic antibiotics [114].

Among HCT recipients, the major benefit of IVIG is the reduction of acute GVHD in allogeneic HCT. The administration of IVIG for the prevention of infections among these patients with severe hypogammaglobulinemia (serum Ig G < 400 mg/dL) is commonly practiced but is of unproven value.

Active Immunization

The immunization of patients with hematological malignancies undergoing cytotoxic chemotherapy has three goals: (a) maintaining the appropriate adult immunization schedule; (b) restoring the immunity that could have been lost after the immunosuppressive treatment; and (c) protecting the patient from the receipt of live vaccines. A suggested schedule for immunization in HCT is shown in Table 49.5.

Table 49.5 Immunization after hematopoietic stem cell transplantation (HCT)

Vaccine	Time after HCT (months)	Number and interval of doses	Comments
Diphtheria, tetanus toxoid, pertussis	6–12	3	Acellular pertussis vaccine preferable
Pneumococcal 7-valent conjugate vaccine	3–6	3	The 7-valent pneumococcal conjugate vaccine is preferable; the polysaccharide vaccine can be given subsequently to broaden the immune response
Pneumococcal 23-valent polysaccharide	12	1	
H. Influenzae type B	6	3	
Meningococcal	6–12	1	Follow recommendations for the general population in the country/region
Hepatitis B	6	3	
Influenza	4–6	1	Repeat every fall
Measles	24	2	All children and posttransplant seronegative adults
Mumps, rubella	24	1	
Inactivated polio virus (Salk)	6–12	3	Inactivated polio vaccine should also be used in household contacts
Varicella vaccine	24	1	Limited data regarding safety and efficacy. Should be given only to seronegative patients

HCT hematopoietic cell transplantation
AVOID live vaccines until patient in complete remission, *and* not receiving immunosuppressive therapy for 6 months *and* has a CD 4 + count >400/μL *and* does not have chronic graft versus host disease
Live vaccines:
– Adenovirus, BCG, Measles-Mumps-Rubella, Oral typhoid, Oral polio, Yellow fever, Varicella zoster
– AVOID oral polio in household contacts (the polio virus may spread and cause uncontrolled infection)
AVOID vaccines until CD 4+ counts > 200/μL (unlikely to be effective)
Consider measuring antibody titers after vaccination to ensure efficacy and repeat doses until optimal titers achieved

Colony-Stimulating Factors (CSF)

Granulocyte colony-stimulating factor (G-CSF) has been shown to reduce the incidence of fever, and duration of antibiotic therapy and hospitalization in some studies. However, a significant reduction of culture-proven infections or mortality

has not been shown. The best cost-effective prophylactic use of G-CSF is in settings when the risk of febrile neutropenia is >20% [115–117].

Granulocyte Transfusions

Prophylactic GM-CSF or G-CSF elicited granulocyte transfusions remains investigational and may be considered in patients with a history of a neutropenia-related invasive mold infection (such as aspergillosis or fusariosis) who are expected to be neutropenic for ≥14 days [118].

Summary

Infection is a frequent complication and a leading cause of morbidity and mortality in patients with hematological malignancies. In general, the higher is the risk for a certain infection, the more beneficial is likely to be prophylaxis. Likewise, the shorter is the period at risk (and therefore the predicted duration of prophylaxis), the higher is the possibility that prophylaxis will work. The decision of giving prophylaxis should take into account its potential benefits, but also side effects, costs, induction of resistance, and the potential for drug interactions with antineoplastic h48 drugs. Risk assessment is a key element in defining prophylactic strategies.

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Evaluation and Management of Bacterial and Fungal Infections in Patients with a Hematological Malignancy: A 2018 Update

50

Maria Pia Franco, Jaime S. Green, and Jo-Anne H. Young

Introduction

The treatment of hematological malignancies relies heavily on cytotoxic chemotherapy that most often places patients at risk for invasive bacterial and fungal infections due to disruptions in mucosal barrier integrity and impaired myelopoiesis. A weakened mucosal barrier permits translocation of fungi and bacteria present in the oropharynx, gastrointestinal tract, or skin to enter the body and, when this is combined with immune compromise from impaired myelopoiesis, severe infections are not uncommon. Antineoplastic therapy-associated neutropenia frequently results in a muted inflammatory response, with fever often being the sole presenting symptom. In addition to cytotoxic antineoplastic therapy, these patients often receive concomitant glucocorticoids which may altogether blunt even a febrile response [1]. This necessitates a high index of suspicion on the part of the clinician to effectively recognize infection in the neutropenic patient who might only present with hypotension or tachycardia with or without other nonspecific findings. Also known as neutropenic fever (these terms will be used interchangeably throughout this chapter), this clinical entity is defined as a single oral temperature measurement of 38.3 °C, or a temperature greater than 38.0 °C that is sustained for more than an hour in a neutropenic patient [2–4]. The generally accepted definition of neutropenia is an absolute neutrophil count (ANC) of <1500 cells/μL. Severe neutropenia is defined as an ANC of <500 cells/μL, or when there is an expected ANC nadir of <500 cells/μL within the next 48 h.

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Bacterial Infections

The pathogenesis of bacterial and fungal infections in the patient with hematologic malignancy is a complex interplay between host factors, effects of antineoplastic therapy, and changes in the host caused by the underlying malignancy (Table 50.1). As mentioned in the introduction to this chapter, the impairment of mucosal barriers and immune system dysfunction create an environment favorable to invasive microbial infections that is often compounded by frequent healthcare exposure and patients' inherent increased risk for exposure to organisms of increased virulence and drug resistance [2]. It is believed that the majority of infections in patients with hematologic malignancies are due to treatment-related muco-

Table 50.1 Risk factors associated with an increased risk of developing febrile neutropenia

Host factors
Age > 65 years
Female gender
Poor nutritional status
Poor performance status based on underlying cardiovascular, pulmonary, and renal comorbidities
Factors related to the underlying malignancy
Advanced stage of the malignancy
Myelophthisis
Lymphopenia
Elevated serum lactate dehydrogenase in patients with lymphoreticular process
Factors related to the antineoplastic therapies
Expected high dose density chemotherapy
Expected intensity of high dose chemotherapy
Lack of administration of prophylactic hematopoietic growth factor stimulants in patients with high-risk chemotherapeutic regimens

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sitis and translocation of gastrointestinal tract flora and colonizing organisms that eventually reach the bloodstream. Less common mechanisms include disruption of respiratory, genitourinary, and lymphatic barriers due to the underlying malignancy or associated with medical procedures. Of particular concern are the presence of central venous catheters (CVCs) and other indwelling catheters [5].

Epidemiology

Bacterial infection is a common cause for febrile neutropenia in patients who have received antineoplastic chemotherapy, with approximately 23% of them presenting with fever or neutropenic fever [6]. Of patients who present with febrile neutropenia, an infectious etiology is only identified in roughly one-quarter of cases, and often only through isolation of an organism causing bacteremia, which is found in up to 25% of all patients with neutropenic fever [2, 7]. Of note, the vast majority of organisms causing infections in hematological cancer patients are bacteria, followed by fungi and viruses as distant second and third place culprits. Given the principal factors that predispose to febrile neutropenia (disruption of mucosal barriers and immune suppression), translocation and infection by saprophytic flora explain the overwhelming predominance of these organisms isolated from cultures [8, 9].

The most commonly isolated organisms tend to be Gram positive bacterial organisms, with a predominance of *Staphylococcus epidermidis*, followed by a variety of other streptococci and staphylococci including *Staphylococcus aureus* [10, 11]. There has been a significant shift in the patterns of causative bacterial pathogens over time due to a variety of factors including antibiotic pressure from prophylaxis and/or treatment, the increased use of long-term indwelling catheters, and novel antineoplastic regimens [12]. Prior to the early 1990s, the majority of organisms isolated during evaluation of episodes of febrile neutropenia were Gram negative bacteria, with *Pseudomonas aeruginosa* being especially notorious [13, 14]. This trend began to turn gradually, with a growing predominance of Gram positives being most commonly isolated in the mid-1990s and into the early 2000s, when Gram negative prophylaxis was common. Approximately 80% of all isolates were Gram positive bacterial organisms [15, 16]. Today a reversal in this trend is being noted, this time the cause for the trend starting to turn back to Gram negative bacterial organisms seems to be related to the emergence of multidrug-resistant organisms (MDROs). MDRO groupings commonly referred to in practice include Gram positive bacteria [methicillin-resistant *Staphylococcus aureus* (MRSA), vancomycin-resistant enterococci (VRE)], as well as Gram negative bacteria [extended spectrum beta lactamase (ESBL), *Klebsiella pneumoniae* carbapenemase (KPC), and carbapenem-resistant enterobacteriaceae (CRE)]. Currently the overall percentage of infections due Gram positive bacterial organisms has decreased to around 60% [17–21]. Another important point is that outside of bloodstream infections, where Gram positive bacterial organisms are predominant, Gram negative bacterial organisms are the most common pathogens of infections related to the urinary, gastrointestinal, and biliary tracts [22].

The severity of bacterial infections varies due to a number of factors specific to each organism. Among the Gram positive bacterial organisms, MRSA and VRE are some of the most severe [16]. Though infrequent, anaerobes can contribute to severe, life-threatening infections such as necrotizing fasciitis, typhlitis (neutropenic enterocolitis), and sinusitis. Interestingly, though still rare, polymicrobial infections also appear to be becoming more frequent than in prior decades [19, 23, 24].

Risk Factors

Cancer patients undergoing systemic chemotherapy for hematologic malignancies often suffer significant side effects, especially effects on mucosal integrity and myeloid production and function. These will predispose to the development of infections due to translocation of gastrointestinal tract colonizing bacteria and fungi. Concomitant neutropenia results in a blunted immune response to invasion by previously colonizing organisms [25]. A thorough evaluation of the host characteristics, risk for developing febrile neutropenia, and risk assessment for serious complications associated with febrile neutropenia will guide empiric antimicrobial therapy and dictate additional workup and need for hospital admission [2]. Risk factors associated with an increased risk of developing neutropenic fever are best thought of in terms of host factors, factors related to the underlying malignancy, and those related to the antineoplastic therapies (Table 50.1). Host factors include the patients' underlying conditions and comorbidities that may alter their immune function (e.g., drug-induced neutropenia will predispose to bacterial and candidal infections, underlying cellular immunodeficiency will predispose to opportunistic viral

infections, and underlying humoral immunodeficiency will predispose to mycobacterial infections), those that predispose to infection due to anatomic and or functional abnormalities (vesicoureteral reflux, nephrolithiasis, bronchiectasis, etc.) and those that alter patients' flora (prolonged antibiotic use that causes selective pressure favoring more invasive or resistant organisms). Treatment-related factors include mucosal barrier disruption due to cytotoxic therapy's effect on high turnover cells of the lining the gastrointestinal tract, impaired myelogenous production, and decreased neutrophil phagocytic and chemotactic activity.

Patients with hematologic malignancy presenting with neutropenic fever should be risk stratified according to their risk of developing serious complications (Table 50.2), which in turn guides the need for hospital admission, parenteral antimicrobial therapy, and prolonged hospitalization (Fig. 50.1).

Table 50.2 Risk of medical complications of febrile neutropenia

Patient Risk Category	National Comprehensive Cancer Network	Infectious Disease Society of America
High	– Developing fever while inpatient	– Anticipated ANC ≤100 cells/μL for >7 days
	– Significant comorbidity or clinical instability	– ALT >5x normal or CrCl <30 mL/min
	– Anticipated ANC ≤100 cells/μL for >7 days	– Concurrent clinical conditions such as: Hemodynamic instability, intravascular catheter infection, encephalopathy, respiratory failure, mucositis or other gastrointestinal symptoms, underlying chronic lung disease and or hypoxia/ infiltrates
	– ALT >5x normal or CrCl <30 mL/min	
	– Leukemia not in complete remission or non-leukemia cancer with evidence of disease progression after >2 courses of therapy	
	– Presentation with pneumonia or other complex infection	
	– Alemtuzumab in prior 2 months	
	– Grade 3 or 4 mucositis	
	– MASCC score ≤ 20	
Medium	– Expected 7 to 10-day duration of neutropenia	
	– Lymphoma	
	– CML	
	– Autologous HCT	
	– Purine analog therapy	
Low	– No High Risk features and most of the following criteria are present:	– Anticipated ANC ≤100 cells/μL for <7 days
	– Outpatient at onset of fever	– Absence of renal and hepatic dysfunction
	– No associated acute concurrent condition requiring hospitalization or close observation	– No comorbidities
	– Anticipated ANC ≤100 cells/μL for <7 days	
	– Absence of renal and hepatic dysfunction	
	– Good performance status (ECOG 0–1)	
	– MASCC score ≥ 21	

Adapted from:Baden LR, Swaminathan S, Angarone M, Blouin G, Camins BC, Casper C, et al. Prevention and Treatment of Cancer-Related Infections, Version 2.2016, NCCN Clinical Practice Guidelines in Oncology. Journal of the National Comprehensive Cancer Network: JNCCN. 2016;14(7):882-913Freifeld AG, Bow EJ, Sepkowitz KA, Boeckh MJ, Ito JI, Mullen CA, et al. Clinical practice guideline for the use of antimicrobial agents in neutropenic patients with cancer: 2010 update by the Infectious Diseases Society of America. Clin Infect Dis. 2011;52(4):e56-93

ANC absolute neutrophil count, *ALT* alanine aminotransferase, *CrCl* creatinine clearance, *MASCC* Multinational Association for Supportive Care in Cancer, *CML* chronic myelogenous leukemia, *SCT* hematopoietic stem cell transplant, *ECOG* Eastern Cooperative Oncology Group

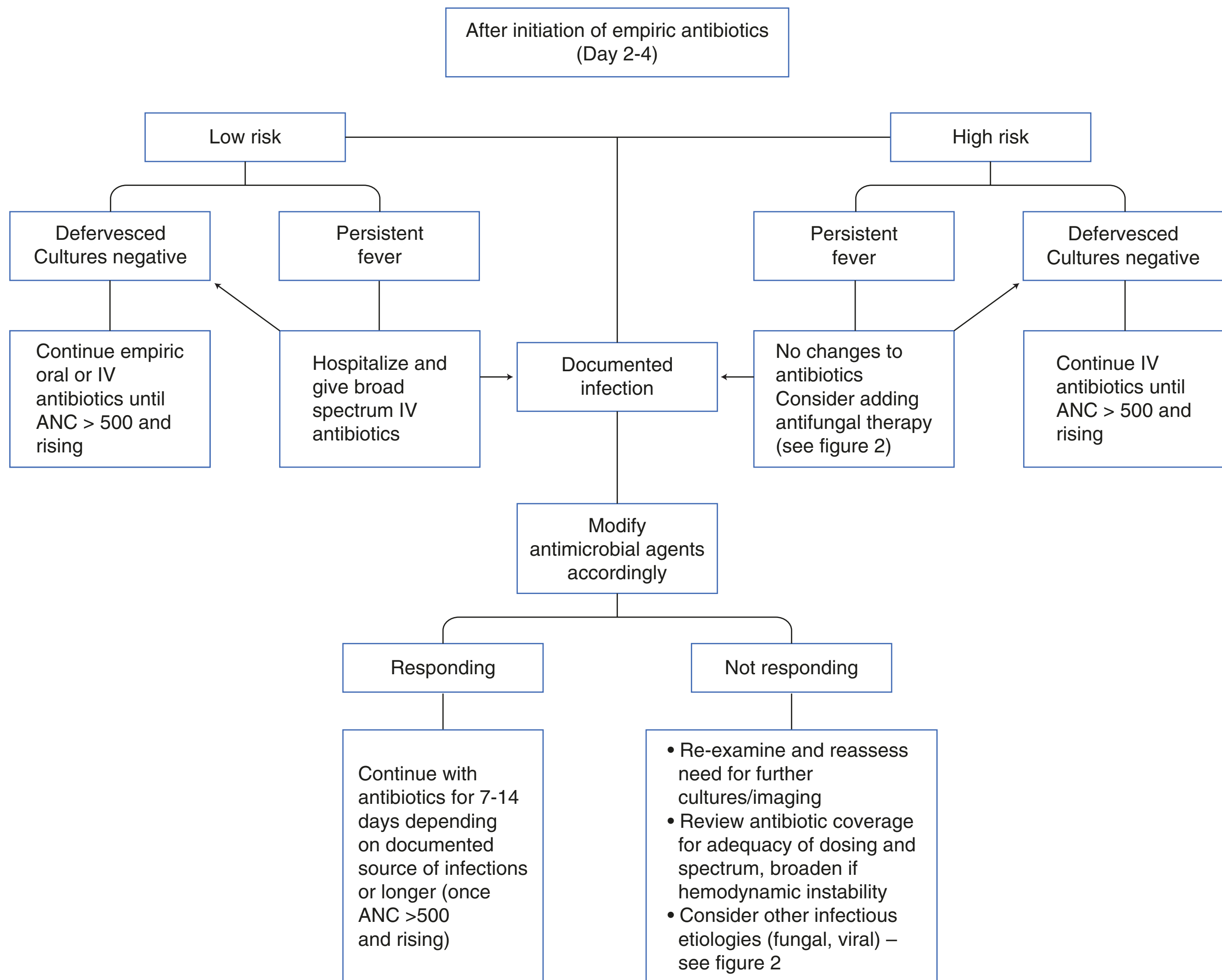

Fig. 50.1 Initial treatment algorithm for febrile neutropenia

Diagnosis

The clinical manifestations of bacterial infections in the patient with hematological malignancies can be very nonspecific due to impaired inflammatory processes. Though much emphasis so far in this chapter has been placed on febrile neutropenia, it is not uncommon for these patients to present with nonspecific findings and be infected despite the absence of fever, or conversely to have a fever as the sole presenting symptom. For the hematological malignancy patient presenting with neutropenic fever, or thought to be otherwise infected, it is imperative to conduct a rapid and thorough workup that focuses on the prompt collection of cultures, especially blood cultures, to enable rapid initiation of broad spectrum empiric antibiotic therapy without compromising their diagnostic yield. The sooner appropriate empiric antimicrobial therapy is initiated the better, as delays in administration correlate with mortality [26, 27].

A thorough history with particular emphasis on pertinent host, underlying malignancy, and antineoplastic therapy factors should be performed in addition to eliciting a listing of careful relevant exposures in the context of the aforementioned factors. The physical exam should be a meticulous head-to-toe examination focused on the skin and mucosal surfaces, oropharynx and teeth, sinuses, heart and lung systems; abdominal, genital and perineal exams should not be overlooked. Peripherally Inserted Central Catheters (PICCs), implanted ports, urinary catheters, old IV sites, procedural sites such as biopsy, aspirate and surgical sites also merit careful visual inspection and palpation. The blunted immune response can manifest in a paucity of inflammatory signs and requires a high index of suspicion.

Peripheral blood counts with white blood cell (WBC) differentials should be obtained as well as at least two sets of blood cultures from the outset when infection is suspected. The hematologic tests serve to both quantify and stratify the

presence and severity of neutropenia, while the blood cultures aim to confirm an infection and identify the causative organism and subsequently determine its antimicrobial susceptibility patterns to tailor anti-infective therapy. When obtaining blood cultures, one should ideally obtain two sets of cultures of 20 mL each in the adult patient. These should be collected from two separate peripheral venipunctures in patients without indwelling venous catheters. In patients with indwelling venous catheters, one set should be from a peripheral venipuncture and one from the indwelling venous catheter. The frequency of subsequent cultures varies depending on the following variables: if fever persists after 24 h on appropriate broad spectrum anti-infective therapy, an additional 2 sets of blood cultures should be obtained and may be repeated a second time after 48 h in the presence of persistent fever. If the fever appears to have defervesced for greater than 48 h and recurs, it is reasonable to repeat blood cultures. Additionally, if initial blood cultures are positive, these should be repeated daily until bacteremia clears regardless of the fever pattern. A study published in 2013 has looked at an alternate single 40 mL sample method from central lines that seems promising, but needs further validation and cost analysis [28].

Treatment

As mentioned throughout this chapter, the prompt recognition and institution of appropriate empiric anti-infective therapy in the potentially infected neutropenic patient with hematological malignancy is life-saving. It is recommended that appropriately selected and dosed antimicrobials are received within an hour of medical contact at a maximum, with administration within 30 min being optimal [23, 26, 29, 30]. The concept of adequate antimicrobial therapy must be highlighted and contrasted with appropriate therapy, and though various somewhat different definitions have been published, we will frame the discussion in terms of the definitions set forth in the joint Infectious Disease Society of America (IDSA) and American Thoracic Society (ATS) guidelines on the management of hospital-acquired, ventilator-associated pneumonia and healthcare-associated pneumonia from 2005 and updated in 2016 [31, 32]. According to these definitions, appropriate antimicrobial therapy is the use of drugs with in vitro activity against the confirmed etiologic agent, whereas adequate treatment implies not only the use of the correct (appropriate) antimicrobial agent, but additionally administering the optimal dose as well as choosing a route for administration that allows for tissue penetration of the drug at the site of infection. In order to provide not just appropriate, but adequate, therapy to the neutropenic patient with hematologic malignancy, host factors as well those related to the underlying malignancy and antineoplastic therapy must be considered in order to determine the patient's risk of developing serious complications of neutropenic fever.

Table 50.3 Key Points: Treatment of bacterial infections

Key points: Treatment of bacterial infections
• Fever may be the only sign of a potentially life-threatening infection in immune-suppressed patients
• Start antibiotics promptly. In high-risk patients, start an antipseudomonal beta lactam agent
• Patients receiving fluoroquinolone prophylaxis should not receive empiric therapy with fluoroquinolones
• Adding vancomycin or an antibiotic with enhanced Gram positive activity empirically is not recommended
• Modifications to empiric treatment should be made based on patient's previous cultures or risks for an infection with a MDRO (e.g., MRSA, VRE, ESBL, CRE, KPC)
• Always tailor antibiotics to the underlying pathogen whenever possible
• Continue with antimicrobial therapy until neutropenia resolves

MDRO multidrug-resistant organisms, *MRSA* methicillin-resistant *Staphylococcus aureus, VRE* vancomycin-resistant enterococci, *ESBL* extended spectrum beta lactamase, *CRE* carbapenem-resistant enterobacteriaceae, *KPC Klebsiella pneumoniae* carbapenemase

In addition to this risk stratification, the patient's drug allergies, prior microbiologic culture data, antimicrobial agent exposures, and the local antibiogram need to be considered [33]. As discussed earlier in the Epidemiology section, the majority of causative organisms in neutropenic fever are Gram positive bacteria, especially skin flora; however, Gram negative bacteria tend to cause more serious clinical disease due to their virulence factors, and they are making a resurgence in terms of their frequency driven in part by the increase in MDROs. Table 50.3 summarizes key points in the treatment of bacterial infections in patients with hematological malignancy.

Those patients who are unable to tolerate oral antibiotics or who are risk stratified into the high-risk category should receive intravenous antibiotics (Fig. 50.1). A number of studies have attempted to find an ideal empiric antibiotic regimen, and no regimen has shown itself to be superior [34–39]. There are a number of acceptable monotherapy options that are as efficacious as combination regimens for empiric therapy in high-risk neutropenic fever syndromes: ceftazidime, cefepime, piperacillin-tazobactam (or other antipseudomonal beta lactam agents), imipenem-cilastatin, meropenem, etc. Combination regimens that use extended spectrum beta-lactams with fluoroquinolones, aminoglycosides, or double beta-lactams have not shown superiority and often increase toxicity [38, 39]. Furthermore, combination regimens that use a second agent to cover Gram positive bacterial organisms do not seem to confer clinical or mortality benefits and also are associated with greater toxicity and bacterial resistance [2, 7, 40–43]. The standard use of vancomycin, linezolid, and other drugs against Gram positive bacteria as part of empiric antibiotic regimens for neutropenic fever should be discouraged. These medications should be reserved for patients with presumed line infections, pneumonia, soft tissue infections, and septic shock.

The importance of knowing the patient's colonization status of resistant bacteria (MRSA, VRE, and other MDROs) and previous culture results cannot be overstated, as many patients with hematologic malignancies are colonized with resistant Gram positive organisms. Once appropriate empiric antibiotic therapy has been instituted in a timely fashion, close attention must be paid to clinical response and the progress of culture (with susceptibility data) to determine adequate therapy. Patients with a persistent unexplained neutropenic fever syndrome, who are clinically stable or improving, do not always require a change in antibiotic therapy for their ongoing fever (Fig. 50.2). Among oncology patients with solid tumors and tumor fever, neutropenic fever usually resolves within 48 h of empiric antibiotics; neutropenic fever among patients with hematologic malignancies can take up to an average of 5 days after initiation of treatment to defervesce [2].

Central line associated bloodstream infections (CLABSIs) are frequently the cause of neutropenic fever in hematologic malignancy patients, and adequate antibiotic administration alone is insufficient to treat these patients, with removal of the offending line being necessary. The exception to this rule is CLABSI due to coagulase negative staphylococci, in which case it may be reasonable to treat through the infection [2]. Catheter removal is recommended for infections with *Pseudomonas aeruginosa*, *Staphylococcus aureus*, fast growing atypical mycobacteria, and *Candida* spp., and with less certainty for other fungal species [2, 44–48]. In these cases, antibiotics should be continued for a minimum of 2 weeks after clearance of bacteremia and removal of the CVC, whichever occurred last. Complicated CLABSIs such as those occurring with deep tissue infections (catheter tunnel tract or port pockets), septic thrombosis or embolisms, endocarditis, or persistent bacteremia (defined as greater than 72 h after initiation of adequate therapy), warrant extended courses of antibiotics in the 4- to 6-week duration range. See Fig. 50.3 for recommendations for management of infected long-term venous access catheters.

In general, the presence of infected material (such as an abscess, a CVC, or a urolith) necessitates both antibiotic penetration and activity to treat the infection. In cases where the drug activity and penetration are suboptimal, removal of the infected material is paramount to achieving control of the infection. Source control requires the removal of infected hardware, drainage of infected fluid collections, or removal of infected tissue or other material. The indications and risk benefit for the interventions already discussed are beyond the scope of this chapter and merit the consultation of infectious diseases specialists, surgeons, or interventional radiologists as clinically dictated.

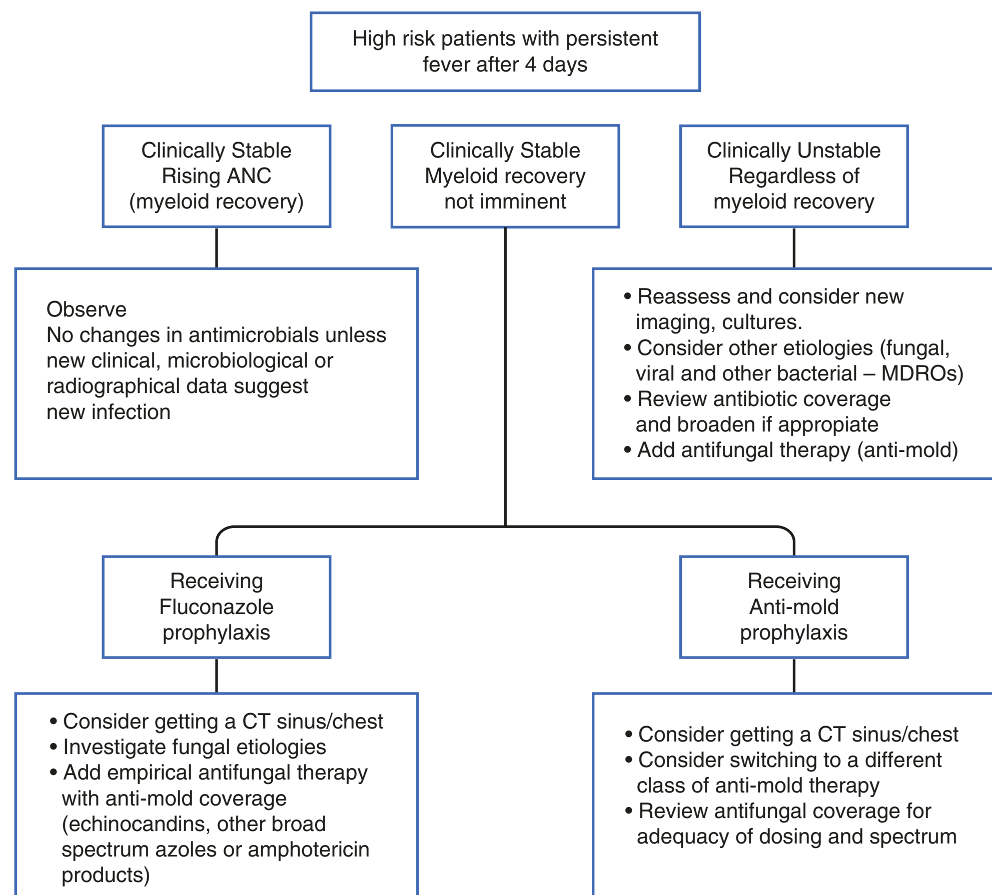

Fig. 50.2 Treatment algorithm for patient with high-risk febrile neutropenia

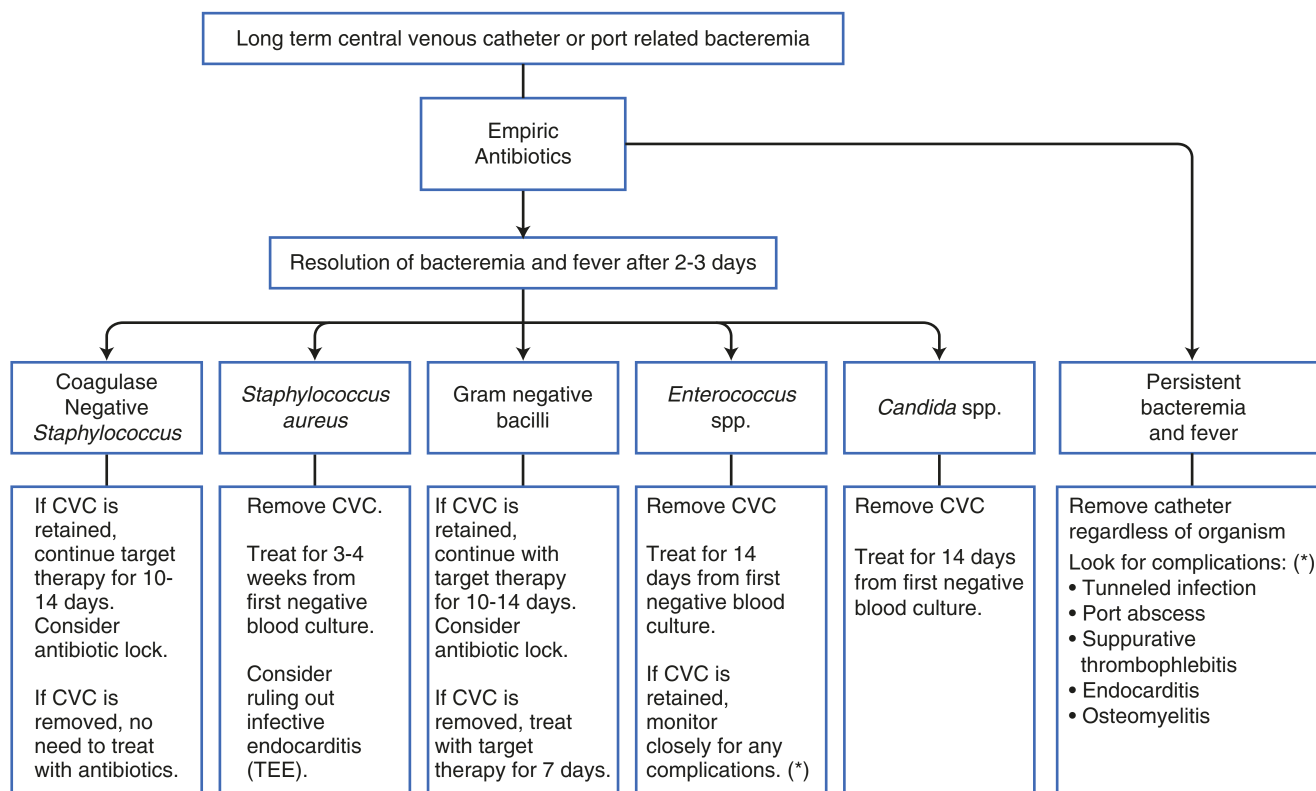

Fig. 50.3 Management of long-term indwelling venous access catheters

Bacterial Prophylaxis

Several studies have demonstrated the utility of antimicrobial prophylaxis to prevent neutropenic fever and infectious complications, especially among high-risk patients [49, 50]. A Cochrane Review meta-analysis in 2012 showed that antibiotic prophylaxis was associated with lower all-cause mortality when compared to placebo or no treatment. Prophylaxis was also associated with significantly reduced occurrence of fever, fewer clinically documented and microbiologically documented infections, and lower risk of infection-related death [49]. Fluoroquinolones (levofloxacin and ciprofloxacin) have been extensively studied given their broad spectrum (covering Gram negative bacteria including *Pseudomonas aeruginosa*, as well as Gram positive bacteria) and good oral bioavailability. Fluoroquinolone prophylaxis reduces the risk for all-cause mortality as well as infection-related mortality [2]. A combination of a fluoroquinolone plus an antibiotic with enhanced Gram positive activity is not recommended [2]. Some studies have shown that this approach may reduce infections caused by *Staphylococcus* and *Streptococcus* spp., but they do not affect infection-related mortality and may increase the rated of resistant bacteria [51–54]. See Table 50.4 for suggested empiric antibiotic regimens for high-risk patients.

There are concerns about toxicities and antimicrobial resistance [52, 55], especially among patients concurrently

Table 50.4 Empiric antibiotic regimens in high-risk patients with febrile neutropenia

Monotherapy options	
Piperacillin/Tazobactam	
Cefepime	
Ceftazidime[a]	
Carbapenems (except Ertapenem since no antipseudomonal coverage)[b]	
Add these antimicrobials based on culture data or specific clinical scenarios	
Vancomycin	If suspected MRSA infection such as cellulitis and/or pneumonia
Linezolid	If suspected VRE infection
Daptomycin	If suspected MRSA or VRE infection, although cannot be used for pulmonary infections as it is inactivated by surfactant
Aminoglycoside/Colistin	If suspected Gram negative MDRO involvement (ESBL, CRE, KPC)
Metronidazole	If suspected intraabdominal infection

[a]Ceftazidime monotherapy has been used for many years, however has less Gram positive coverage than cefepime
[b]Use Carbapenems for patients at high risk of MDROs
MRSA methicillin-resistant *Staphylococcus aureus, VRE* vancomycin-resistant enterococci, *MDRO* multidrug-resistant organisms, *ESBL* extended spectrum beta lactamase, *CRE* carbapenem-resistant enterobacteriaceae, *KPC Klebsiella pneumoniae* carbapenemase

using QT prolonging medications such as amiodarone or voriconazole. In 2016, the Food and Drug Administration (FDA) issued safety warnings about fluoroquinolones causing acute tendonitis, neuropathy, and central nervous system side effects, which has led to a revision of the package inserts for these medications.

The optimal timing for initiation and duration of antimicrobial prophylaxis has not been well studied. Some clinicians will start prophylaxis on the first day of cytotoxic chemotherapy even for those patients not yet neutropenic, while others will do so on the last day of chemotherapy. It is common to discontinue prophylaxis when the neutropenia has resolved.

Fungal Infections

Invasive fungal infections (IFIs) cause significant morbidity in patients with hematological malignancies and stem cell transplants. This section will elaborate on the epidemiology, clinical syndromes, diagnosis, and treatment of the most common IFIs in this population. IFIs are categorized into those caused by yeasts or molds, of which a basic microbiologic understanding is useful when we think about the syndromes and the term "mold active" antifungal agent. Yeasts are single cell organisms that reproduce by budding and include genera such as *Candida* or *Cryptococcus*, among others. Mold organisms have a filamentous growth stage that allows them to elongate by branching with longitudinal extension, and can be further categorized as opportunistic or endemic infections. The morphology of filamentous hyphae on pathology specimens plays an essential part in diagnosis and early management of mold infections. The most common opportunistic mold organisms include *Aspergillus*, *Mucor*, *Rhizopus*, and *Fusarium* species, while *Histoplasma*, *Blastomyces*, and *Coccidioidomyces* species comprise most of the common endemic fungi.

Candida

Candida yeasts are saprophytic organisms commonly found on the skin and mucosal surfaces of humans. Infections with these organisms can cause superficial (oral thrush, esophagitis, dermatitis, and vaginitis, etc.) and/or deep infections (candidemia, visceral organ abscesses, endophthalmitis, and many others). Herein we focus on the invasive forms of candidiasis (IC), which cause significant morbidity and mortality among hematologic cancer patients and stem cell transplant (SCT) recipients. Prior to the use of antifungal prophylaxis, the incidence of disseminated candidemia was approximately 17% among patients with hematologic malignancies [56, 57] and 11% after SCT [58, 59]. Mortality for patients with IC ranged from 39 to 73% [58, 59]. Autopsy studies in SCT patients from the 1980s suggest overall prevalence of *Candida* infections was 28% [60]. Table 50.5 summarizes key points in the management of *Candida* infections.

Table 50.5 Key points: *Candida* spp.

Key points: *Candida spp.*
• The incidence of non-albicans *Candida* is more common than *C. albicans*.
• Non-albicans *Candida* organisms are frequently fluconazole resistant (*C. krusei* and *C. glabrata*)
• Empiric therapy for yeast identified in the blood is an echinocandin, which is then de-escalated pending species identification

Risk Factors and Epidemiology

In addition to the risk factors for IC in the general population (CVCs, broad spectrum antibiotics, and total parenteral nutrition) [61], those with hematologic malignancies are at increased risk of disseminated disease secondary to prolonged neutropenia [62–64], use of antibacterial antibiotics [62, 64], *Candida* colonization [19, 52, 60, 65, 66], and mucosal damage from cytotoxic chemotherapy [62]. Specifically, neutropenia lasting over 15 days is a risk for hepatosplenic candidiasis [62].

Over time the incidence of infection by non-albicans *Candida* spp. is increasing [67, 68], and even surpassing the incidence of *Candida albicans* infections in the United States [68–70]. Hematology patients are at increased risk for specifically *C. glabrata* [63, 67], *C. krusei* [63, 71, 72], *C. tropicalis* [72, 73], *C. guilliermondii* [72], and emerging *Candida* spp. such as *C. dubliniensis* and *C. kefyr* [72]. This evolution of epidemiology is important as these species can be resistant to fluconazole and to a lesser extent voriconazole [72].

Clinical Presentation

The most common clinical presentation of IC in hematologic malignancy patients is fever. Acute disseminated candidiasis can result in severe sepsis and multiorgan failure. Early descriptions of IC from autopsy studies in the 1980s (prior to the use of antifungal prophylaxis in the 1990s) in subjects with acute leukemia report fever refractory to antibiotics or a second episode of fever. At autopsy, often multiple organs were involved [65, 74]. By the late 1990s fever was being reported in 99% patients with malignancies and was most often it was low grade [75].

Chronic disseminated candidiasis or hepatosplenic candidiasis deserves special attention as this entity occurs almost exclusively among patients with acute leukemia. Epithelial damage that occurs along the gastrointestinal mucosa from cytotoxic chemotherapy enables *Candida* to enter the hepatobiliary circulation, resulting in hepatic and splenic microabscesses. Symptoms of disease develop later into the course

of infection, usually just after neutrophil recovery, when neutrophils can migrate to the sites of the microabscesses. As with acute IC, persistent fever is the most common manifestation, sometimes accompanied by right upper quadrant abdominal pain, tenderness, and an elevated alkaline phosphatase. An immune reconstitution-like syndrome has been hypothesized given the timing of symptoms following neutrophil recovery [76].

Diagnosis

There is no true gold standard for the diagnosis of IC, and limitations of culture and non-culture diagnostic tests make this challenging. While diagnosis often relies on positive blood cultures, and blood culture technology has improved over the decades, overall sensitivity of blood culture has been estimated to remain approximately 50% [77]. IC can be divided into three groups: candidemia in the absence of deep-seated infection, candidemia associated with deep-seated infection, and deep-seated candidiasis that is not associated with candidemia. Blood culture systems, while as sensitive as PCR in vitro, may capture 75% of group 1 and 2 above, leading to an overall sensitivity of 50% [77]. The 2- to 3-day turnaround time of blood cultures often delays diagnosis [78]. Of note, in hepatosplenic candidiasis (the third group described above), blood cultures may be positive in only 20% of cases, with tissue culture positivity in approximately 50%.

Non-culture-based techniques include antigen/antibody testing, β-D-glucan, and PCR, which can be used adjunctively with blood cultures. There are several limitations of these non-culture-based tests, some of which include the rapid clearance of antigen/antibody from the circulation and limitations in immunocompromised hosts. β-D-Glucan (a component of the fungal cell wall) is not specific for *Candida*, has poor specificity, and false positivity among immunocompromised patients who are at risk for a number of fungal pathogens. PCR is problematic in that there is a lack of standardization in methodologies and has similar limitations to β-D-glucan testing. Currently these tests are not considered standards in making the diagnosis of invasive candidiasis [77, 78]. There are no guidelines for interpreting these non-invasive diagnostic tests, because they provide adjunctive information to blood cultures and should not be ordered in place of blood cultures.

T2Candida is a newer diagnostic modality that is being implemented into some clinical practices. T2 diagnostic testing involves a miniaturized, magnetic resonance-based diagnostic approach that measures how water molecules react in the presence of magnetic fields. The T2Candida Panel test seems to be capable of improved sensitivity compared to blood culture, using automated systems with a turnaround time of 3–5 h [79]. In some instances, the T2Candida Panel test may be positive up to a week prior to blood culture [79].

Treatment

Treatment of ICs has been extensively reviewed and updated by the IDSA in 2016 [78]. Due to the increase in non-albicans infections, with the potential for fluconazole drug resistance, it has become standard practice that echinocandins (either anidulafungin, caspofungin, or micafungin) are initiated empirically upon microbiologic diagnosis of candidemia until speciation is known. Fluconazole is used in the majority of *C. albicans* infections given the >95% sensitivity of isolates to this drug. Treatment durations range from 2- to 4-weeks on average, depending on the underlying disease [78]. Dilated retinal exams are recommended on all patients with candidemia, but can be delayed until neutrophil recovery in hematologic patients. Removal of CVCs is also widely implemented in clinical practice.

Antifungal Prophylaxis

With the advent of a well-tolerated antifungal drug (fluconazole), initial studies from the 1990s showed that prophylactic fluconazole significantly reduced *Candida* colonization [56], reduced invasive *Candida* infections [80], and decreased the incidence of superficial and systemic IC among SCT recipients, with improvements in survival [81, 82]. A randomized placebo-controlled trial of fluconazole prophylaxis in neutropenic cancer patients identified that patients who benefited most from prophylaxis included those with acute myeloid leukemia undergoing induction with anthracycline-based regimens and those receiving autologous transplants not supported with hematopoietic growth factors [57]. Low dose (200 mg daily) of fluconazole was found to be as efficacious to high dose (400 mg daily) in preventing candidal infections among SCT recipients [83]. Since those pivotal studies, the use of fluconazole prophylaxis has become standard protocol throughout many hematologic and transplant centers and is supported by the National Comprehensive Cancer Network (NCCN) guidelines. Meta-analysis studies of antifungal prophylaxis in neutropenic chemotherapy recipients [84] and stem cell transplant recipients [85] report reduced morbidity, superficial and invasive fungal infections, and fungal infection-related mortality with the use of antifungal prophylaxis [84, 85].

Opportunistic Molds

The term invasive fungal disease (IFD) was coined by the European Organization for Research and Treatment of Cancer (EORTC) Invasive Fungal Infection Cooperative Group and the National Institute of Allergy and Infectious Diseases Mycoses Study Group (NIAID-MSG) in 2008 to describe any disease process dealing with fungi in high-risk patients [86]. The IDSA has now expanded their guidelines

to include other groups of patients at risk such as solid organ transplant and those with primary immunodeficiencies [87]. For the purposes of this section we will refer to IFD as it relates to pathogenic mold fungal infections among hematologic patients, and the discussion will be limited to *Aspergillus*, the agents of Zygomycetes infections (*Mucor*, *Rhizopus,* and *Rhizomucor*) and *Fusarium*, the most common mold infections.

These fungi are saprophytic environmental molds that propagate on decaying soil. Concomitant environmental exposure and host immune deficiency are necessary for invasive disease, of which inhalation of fungal spores is the most common portal of entry. These molds grow by longitudinal extension and branching of filamentous hyphae and are angioinvasive. The morphologic appearance on pathology specimens is pivotal in diagnosing these infections (i.e., *Aspergillus* are seen as branching acute angle septate hyphae, while Zygomycetes are seen as non-septate, broad, ribbon-like hyphae).

Common Features of Invasive Fungal Diseases

There is significant overlap in the clinical and radiographic features of *Aspergillus*, Zygomycetes, and *Fusarium*. Early descriptions of invasive aspergillosis (IA) included persistent fever with pulmonary infiltrates in patients with acute leukemia [74, 88, 89], which also commonly occurs in Zygomycetes [90] and *Fusarium* [91, 92]. The range of pulmonary symptoms includes cough, hypoxemia, shortness of breath, pleuritic chest pain, and (given the angioinvasive character of these fungi) hemoptysis. Risk factors for infection have been extensively studied and include prolonged neutropenia, corticosteroids, and acute leukemia [89, 93–96]. In addition, those patients with active malignancy, persistent neutropenia at the end of treatment, and delayed initiation of treatment are associated with poor survival [97–99]. Delayed therapy for ≥10 days and 6 days has been associated with worse survival in *Aspergillus* [100] and Zygomycetes [99] infections, respectively. Utilization of high-resolution chest computed tomography (CT) scans has allowed earlier diagnosis of IFDs and hence improvement in outcomes with initiation of antifungal therapy.

After entry into the lungs, these mold infections can spread by either direct extension or systemic dissemination via the blood system and angioinvasion [101]. Pulmonary disease is the most common manifestation of infection [102–104], with lung involvement occurring in 90% of cases in one single center study [105]. IA does have a predilection to cause central nervous system disease, either through hematogenous dissemination or contiguous spread from the sinuses, however dissemination into any organ can occur [101]. Table 50.6 summarizes key points regarding invasive mold infections.

Table 50.6 Key points: Invasive fungal diseases

Key points: Invasive fungal diseases
• Invasive aspergillosis is the most common invasive fungal disease among immune-compromised patients
• Rhino-cerebral disease more commonly occurs in Zygomycetes infections
• Ribbon-like non-septate hyphae are characteristic of Zygomycetes histopathology
• Skin manifestations commonly occur in disseminated *Fusarium* infections

Epidemiology

Aspergillus

Invasive aspergillosis is the most common of the opportunistic mold infections occurring in patients with hematologic malignancies and SCT. The actual overall incidence of IA is difficult to estimate; however, the use of non-culture-based diagnostic tests has expanded the number of patients who will be defined as “possible” cases [106]. Among all causes of IFDs among immunocompromised and immunocompetent patients, IA represents approximately 70% of mold infections, with 85% of those occurring in patients with hematologic malignancies and SCT recipients [107]. The majority of IA occurs during the first course of induction chemotherapy for those with AML [108], when mold-active prophylaxis is not routinely employed. A bimodal distribution of IA has been described after SCT, with early disease being related to underlying myeloid deficiencies and late disease (after day 100) related to graft-versus-host disease, steroids, and immunosuppression [108]. *Aspergillus fumigatus* is the most common species isolated [102, 104, 107, 109], followed by *A. flavus*, *A. niger*, and *A. terreus* [102]. A history of aspergillosis during chemotherapy for AML does not preclude progression to transplantation, but the infection should be well controlled unless activity of the underlying transplant condition is the reason why aspergillosis cannot be controlled.

Survival and mortality vary significantly depending on the study and have been reported as 59% survival after hematologic malignances at 12 weeks [102], to 43–62% with hematologic malignancy or after SCT [102, 105, 110]. Factors for poor survival include SCT, progression of underlying disease [111, 112], steroid use [110, 113], neutropenia [112, 113], disseminated disease [113], and extent of pulmonary lesions [105].

Zygomycetes

Common Zygomycetes molds include organisms in the genera *Mucor*, *Rhizopus*, *Rhizomucor*, *Lichtheimia* (formerly *Absidia*), and *Cunninghamella*. Among hematologic malignancy patients, the most common presentation mimics IA; however, Zygomycetes infections do have a greater propensity

to have sinus or rhinocerebral involvement [114–116]. The presence of sinus disease favors Zygomycetes, but does not rule out IA. In the two largest prospective studies on Zygomycetes, the incidence of pulmonary disease ranged from 30 to 46%, with rhinocerebral disease occurring in 27–29% [115, 116]. Single center studies prospectively comparing IA to Zygomycetes infections in those with hematologic malignancies report that sinusitis and rhinocerebral manifestations were significantly more common in those with Zygomycetes infections compared to *Aspergillus* [96, 99]. Other independent predictors for Zygomycetes also included prior use of voriconazole [96, 114], the presence of multiple pulmonary nodules (≥10 nodules), and pleural effusions [114]. Taken together, the two most significant distinguishing factors for Zygomycetes infections include sinus disease and breakthrough IFD on voriconazole prophylaxis.

Worse outcomes have been associated with active malignancy [97, 99], delay in therapy [99], and pulmonary and disseminated disease [93, 116]. A single center retrospective study showed that delay in initiation of an amphotericin product resulted in a twofold increased mortality at 12 weeks [99], which has been confirmed in other studies [115]. Use of posaconazole [99] and neutrophil recovery are associated with favorable outcomes [90, 99]. Mortality ranges from 52% in those with hematologic malignancies to 76% after stem cell transplant [116]. Interestingly, in one small pilot study, radiographic improvement was not predictive of 90-day survival, and 50% who survived did not have improved CT or magnetic resonance imaging at the end of treatment. However, clinical response 30 days after the end of treatment was predictive of survival at 90 days after treatment [97].

Fusarium

Notable characteristics that distinguish *Fusarium* from the above infections is the propensity for disseminated disease (not just pulmonary involvement), skin manifestations that occur in approximately 60% of infections, and the ability to grow in blood cultures in 40–55% of cases [91, 98]. The pulmonary nodules that occur in *Fusarium* infections tend to have more peripheral involvement compared to Zygomycetes or *Aspergillus* infections [117].

Fusarium solani is the most commonly reported isolate representing >50% of the *Fusarium* cases reported in the literature [92, 95, 98]. The most common skin manifestation is multiple painful erythematous papular or nodular lesions with or without central necrosis, and can also manifest as ulcerations, bullae, or ecthyma gangrenosum [118]. Skin lesions have been described to precede fungemia by 5 days [95, 119]. Early descriptions of *Fusarium* in hematologic patients reported fever refractory to antibacterial therapy with painful skin lesions in 91% of infections, with presumed pneumonia and sinusitis occurring in 84% and 26%, respectively [92].

Diagnosis

The EORTC/MSG and IDSA all support a composite definition for the diagnosis of IFD that includes host factors, clinical manifestations, and mycologic evidence of infection. Utilizing these criteria, patients are grouped as either proven, possible or probable IFD, with proven infection necessitating tissue biopsy with pathologic evidence of fungi invading tissue. Probable is the most common category in clinical practice, which includes a host factor (such as allogenic SCT, corticosteroids, T cell immune-suppression, or recent history of neutropenia >10 days), clinical features (such compatible CT imaging), and mycologic evidence (direct or indirect mycologic testing) be present [86].

Use of high-resolution CT imaging and non-culture-based diagnostic testing improves early diagnosis of IFD in hematologic patients, which in turn impacts outcome. There are several classic patterns of IFD on high-resolution CT of the lung, including the halo, air-crescent, or reverse halo sign [120]. The macronodule is the most common manifestation of IA [121]. A macronodule surrounded by ground glass (the halo sign) pathologically represents an area of fungal tissue invasion surrounded by alveolar hemorrhage [87, 120, 122]. The halo sign occurs early in the course of IFD, and while not specific for IA, it commonly occurs among hematologic patients with invasive aspergillosis (92–95% of patients in small series) [120, 123, 124]. The reverse halo sign, a focal area of ground glass opacity surrounded by a ring of consolidation, also occurs early in the course of IFD, but is more associated with infections caused by the agents of Zygomycetes rather than *Aspergillus* [120]. As neutrophils recover, cavitation and gas formation may occur, denoting the air-crescent sign.

Treatment

Azole antifungal agents (fluconazole, itraconazole, voriconazole, posaconazole, and isavuconazole) are the most commonly used class of agents used to treat IFD infection, given their improved tolerability profile over amphotericin products. Table 50.7 expands upon the different antifungal agents, with expanded coverage of the higher generation azole antifungal agents. Echinocandins (micafungin, caspofungin, and anidulafungin) have good *Aspergillus* coverage, but lack activity against the agents of Zygomycetes and *Fusarium*. These are often used along with an azole for dual drug or salvage therapy for *Aspergillus*. Finally, amphotericin products do have a broad spectrum, but are most commonly used to treat Zygomycetes infections. Nephrotoxicity and infusion-related reactions are the most common limiting factors in using these agents. Knowledge of the antifungal spectrum that is used for prophylaxis is essential in making early treatment choices for suspected IFDs and breakthrough fungal infections.

Therapy for *Aspergillus*: In 2002 a randomized clinical control trial showed better treatment responses and improved

Table 50.7 Key points: Commonly used Antifungal agents

Class of antifungal agent	Route of administration	Individual antifungal agent	Characteristic
Azole (Liver metabolism effects)	Water soluble	Fluconazole	– Most activity with *Candida albicans* and *Candida parapsilosis*
	IV and enteral		
		Voriconazole	– Predictable visual side effects
		Isavuconazole	– Zygomycetes activity
			– Administered as a prodrug
	Lipid soluble	Itraconazole	– Absorption variable
	For the most part, used enterally		– Enteral agent only
		Posaconazole	– Zygomycetes activity
			– Extended release form with reliable absorption
Echinocandin	IV only	Anidulafungin	– None of these agents cover *Cryptococcus* or Zygomycetes
		Caspofungin	
		Micafungin	
Polyene	For the most part, used IV	Amphotericin products	– Zygomycetes activity
			– Considered fungicidal
			– Nephrotoxicity common
			– Infusion-related side effects
		Nystatin	Topical agent only

IV intravenous

survival with voriconazole as compared to amphotericin-B [109], which has been confirmed by two superseding studies [105, 113]. Given the high mortality of IA in immune-compromised patients, combination antifungal therapy is often considered for patients with a high risk of death and for critically ill patients. This usually consists of voriconazole plus an echinocandin. A randomized, controlled trial of voriconazole monotherapy versus combination therapy was published in 2015 [125]. There was a trend toward improved mortality at 6 weeks with combination therapy in patients with hematological malignancy or SCT, although statistical significance was not obtained.

Therapy for Zygomycetes: Mold active agents with activity against Zygomycetes include amphotericin, posaconazole, and isavuconazole. Double coverage of amphotericin and posaconazole is the most common regimen used early in the course of therapy. Surgical debridement in those with sino-cerebral disease is an important part of therapy and improves outcomes [90, 93, 126]. Hyperbaric oxygen and granulocyte transfusions are often considered in those with severe disease.

Therapy for *Fusarium*: The most common regimens for therapy include voriconazole with or without amphotericin, and therapy should be based on the isolate's sensitivities. Neutrophil recovery is essential for recovery from *Fusarium* infections [63, 91], with some series reporting 0% survival without recovery from myelosuppression [92]. Granulocyte transfusions can be considered as an adjunct to antifungal therapy, working as a bridge until recovery from myelosuppression. Steroid use is also associated with poor outcome [91].

Summary

The evaluation of potential bacterial and fungal infections in patients with hematological malignancies necessitates a careful understanding of host, underlying disease, and anti-neoplastic therapy factors. The likelihood of infections that one is likely to encounter will influence the types of diagnostic testing and empiric treatment that should be ordered. Additionally, empiric and targeted treatments are influenced by the current knowledge of local microbial prevalence and antimicrobial resistance patterns. Providers managing these patients need to have a good understanding of these concepts and are encouraged to involve infectious diseases experts in the management of these complex patients, especially when facing poor clinical response to anti-infective therapy, MDROs, and fungal infections. To be certain, this is a constantly changing field, if nothing else due to microorganisms' ability to constantly evolve and adapt; therefore, we must remain flexible, observant, and willing to adapt as well.

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Viral Infections in Patients with Hematological Malignancies

51

Jack W. Hsu, John W. Hiemenz, John R. Wingard, and Helen Leather

Introduction

Enormous advances in the control of bacterial and fungal pathogens have occurred over the last decade. Although host factors such as impairment of granulocyte function or impaired mucosal barrier integrity are major risk factors for bacterial and fungal pathogens, compromise of cell-mediated immunity, especially of cytotoxic thymus-derived lymphocytes (T cells), appears to be most significant in accounting for susceptibility to viral pathogens. As dose intensity of the chemotherapeutic regimens used in the therapy of hematological malignancies has escalated over the last decade, greater susceptibility to viral pathogens has emerged.

Patients receiving therapy for leukemia or lymphoma and those undergoing bone marrow transplantation are susceptible to a variety of viral pathogens (Table 51.1). The most significant viral infections are caused by members of the herpesvirus family. In recent years there has also been increasing recognition of important manifestations of other viruses. In this chapter various viral infections will be described, and current approaches to treatment and prevention will be reviewed.

Herpesvirus Family

The herpesviruses are large double-stranded DNA enveloped viruses, which commonly infect humans. Typically, the primary infection is mild and self-limited and requires no therapy in the normal host. The human herpesviruses that cause infection are herpes simplex virus type 1 (HSV-1), herpes simplex virus type 2 (HSV-2), varicella zoster virus (VZV), cytomegalovirus (CMV), Epstein–Barr virus (EBV), and human herpesvirus type 6 (HHV-6). Human herpesvirus type 7 (HHV-7) has also been isolated, but its role in illness has not been elucidated. Human herpesvirus type 8 (HHV-8) gene sequences have been identified in tissue specimens of Kaposi's sarcoma and some types of non-Hodgkin's lymphoma, and an association with multiple myeloma has also been proposed.

Following resolution of the manifestations of the primary infection, lifelong latency can occur in normal hosts; the sites of latency are sensory nerve ganglia for HSV and VZV and leukocytes for CMV, EBV, and HHV-6. Endogenous virus can subsequently cause a reactivated infection in both normal and immunocompromised hosts. Generally in the normal host, reactivated infections have milder clinical symptomatology. All have been implicated as pathogens in patients with hematologic malignancies with the capacity to cause more severe clinical manifestations.

Herpes Simplex Virus

Patterns of Infection

Infections with HSV-1 frequently occur in early childhood. Transmission of virus occurs through contact with oral secretions. Transmission of HSV-2 occurs through contact with infected genital secretions, and thus infection generally occurs later in life, after adolescence. Clinical manifestations of HSV-1 include oral or nasolabial vesicles or ulcerations, keratoconjunctivitis, or encephalitis, whereas HSV-2 primarily causes genital vesicles or ulcerations. Serological conversion occurs with the primary infection, and the presence of IgG antibody provides an excellent indicator of prior infection and the presence of latent virus.

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Table 51.1 Viral pathogens in patients with hematologic malignancies

Herpesviruses
Herpes simplex type 1
Herpes simplex type 2
Cytomegalovirus
Varicella zoster virus
Epstein–Barr virus
Human herpesvirus 6
Intestinal viruses
Rotavirus
Norwalk virus
Adenoviruses
Astroviruses
Coxsackie
Caliciviruses
Respiratory viruses
Respiratory syncytial virus
Influenza
Parainfluenza
Metapneumovirus
Adenoviruses
Hepatitis viruses
Hepatitis A
Hepatitis B
Hepatitis C
Papovaviruses
JC
BK
Human papilloma
Other
Parvovirus
HTLV/HIV
West Nile

Immunocompromised patients are more susceptible for reactivation and potentially more severe manifestations [1] than normal hosts. In general, the intensity of chemotherapy regimens used in the treatment of hematological malignancy and the depth of depression of cell-mediated immunity correlate with both the risk for reactivation and the severity of the resulting clinical manifestations (Table 51.2). In seropositive bone marrow transplant (BMT) patients, the risk for recurrence is 70–80% [2, 3]. In adult acute leukemia patients undergoing induction therapy, there is a similar 60–70% frequency of reactivation [4]. In patients receiving ambulatory chemotherapy regimens for non-Hodgkin's lymphoma, the risk is lower, in the range of 40–50% [5, 6]. For comparison purposes, in patients receiving outpatient regimens for solid tumors (which are generally much less intensive than regimens used in the treatment of hematological malignancies), the recurrence rate is generally less than 10–25% [7, 8].

Table 51.2 Rates of herpes simplex virus type 1 (HSV-1) and varicella zoster virus (VZV) infections in different patient groups

	Reactivation	
Group	Rates of HSV-1 infection (%)	Rates of VZV infection (%)
Bone marrow transplant	70–80	20–50
Acute leukemia	60–70	30
Hodgkin's lymphoma	40–50	15–20
Non-Hodgkin's lymphoma	40–50	5–10
Solid tumors	10–25	5

Diagnosis, Prophylaxis, and Treatment

The manifestations of HSV-1 infection in patients with hematological malignancies are generally intraoral. Frequently, the labial lesions typically seen in the nonimmunocompromised host are absent and the oral ulcerations are indistinguishable from stomatitis due to tissue damage from cytoreductive therapy; thus, this distinction can pose a difficult diagnostic challenge. The concomitant occurrences of HSV infection and chemotherapy-induced mucosal damage result in a more severe form of mucositis [9]. The lesions tend to be larger, slower to heal, and more apt to become secondarily infected by bacterial opportunists. Atypical cutaneous satellite lesions may occur by autoinoculation of infected oral secretions into cutaneous abrasions. Extensive and deep oral ulcerations can lead to a greater susceptibility for bacteremia by α-streptococci, especially *Streptococcus mitis*, an organism that normally resides on the buccal mucosa [10]. Typically, HSV-2 infections cause vulval, intravaginal, or perianal ulcerations or vesicles.

Although the oral and genital mucosa are the primary sites of HSV infections, in the immunocompromised host extension to the esophageal and tracheal mucosa may occur [11–15].

Esophagitis can be caused by HSV alone or as part of a polymicrobial infection (especially Candida and CMV) [12, 16]. Endoscopic biopsy is important to distinguish various etiologies. Dissemination and involvement of visceral tissues can occur in profoundly immunocompromised patients [13–15, 17–19]. Herpesvirus pneumonia was noted to account for up to 5% of nonbacterial pneumonias among allogeneic BMT patients [18, 20] in the preantiviral era.

Cultures of infected secretions or lesions permit confirmation of the diagnosis. Rapid methods using the shell vial technique coupled with antigen detection procedures [21] or using antigen detection immunofluorescent or immunoperoxidase assays alone [22–24] offer quicker and easier alternatives but may be less sensitive or specific. Cytologic examination of cells scraped from lesions using the Tzanck procedure can show the multinucleated giant cells caused by

herpetic infection; this is rapid but relatively insensitive and cannot distinguish HSV from VZV. Detection of viral DNA by polymerase chain reaction (PCR) is an alternative to culture with similar sensitivity and specificity [25]. Although as noted serology can identify patients at risk for reactivation, it is not of value for the diagnosis of infection.

Typically the lesions from HSV infection occur 7–21 days after initiation of chemotherapy. This association with active therapy and its predictable temporal occurrence in seropositive patients has led to the development of prophylaxis strategies, as discussed next. In patients who are not being actively treated but who have severe deficiency of cell-mediated immunity due to progressive disease, malnutrition, cachexia, use of high-dose corticosteroids, or severe graft-versus-host disease (GvHD) after marrow transplantation and in patients with the acquired immune deficiency syndrome (AIDS), infection can occur at any time, and chronic progressive infections may occur.

Acyclovir, a purine analogue, is highly active against HSV types 1 and 2. Its phosphorylation by a viral-encoded thymidine kinase is necessary for its activation. Its poor phosphorylation by homologous cellular enzymes offers antiviral specificity. Further phosphorylation by cellular enzymes then occurs. Inhibition of the viral-encoded DNA polymerase provides additional antiviral specificity and accounts for its selective antiherpes activity. Finally, the triphosphorylated acyclic nucleoside is incorporated into the viral DNA chain, leading to chain termination and production of a defective virion.

Acyclovir has been demonstrated to be highly effective in both prophylaxis and treatment of HSV infections in patients with hematologic malignancies (Table 51.3). A variety of oral and intravenous regimens have been evaluated and found to be effective [3, 4, 26–32]. In treatment studies, shortening of the duration of viral shedding, time to pain relief, and interval until healing of lesions have been demonstrated. In prophylaxis studies, excellent control with few breakthrough infections has been noted. Because of its poor bioavailability (only 15% after oral administration), caution should be exercised in patients who may have difficulty in tolerating oral medications, such as patients with severe mucositis or gastrointestinal toxicity, in order to ensure adequate plasma concentrations. Acyclovir is generally well tolerated but may be associated with nephrotoxicity if patients are not adequately hydrated or infusions are given too rapidly. Rare reports of reversible neurotoxicity, including tremulousness, lethargy, agitation, and disorientation, have been reported; these toxicities resolve with discontinuation of the drug [33, 34].

Valacyclovir, the L-valyl ester of acyclovir, is a prodrug that is metabolized to acyclovir within minutes of oral administration [35]. The bioavailability of acyclovir with this prodrug is approximately 50% [36]. Plasma acyclovir levels are substantially higher and approximate those achieved with intravenous acyclovir [37]. Less frequent dosing is thus possible.

It is important to note that acyclovir is virustatic and suppresses viral replication only during the interval of drug administration. Following cessation of acyclovir, reactivation frequently occurs. When used in neutropenic leukemia and BMT patients, a course of therapy is generally given until neutrophil recovery. Recurrences after neutrophil recovery tend to be milder and self-limited and may not require retreatment. Often in nonneutropenic patients, treatment is given for 10–14 days. If severe immunodeficiency persists, lower dose maintenance therapy is sometimes advocated to prevent recurrence after the lesions are healed, to be continued until immunity is more robust.

There is considerable debate as to the relative merits of prophylaxis versus waiting until an established infection is documented before initiating treatment. Factors to be weighed in choosing between prophylaxis and treatment include the likelihood of infection, the depth of host immune deficiency, and the severity of infection if it occurs. Thus, while prophylaxis might be an acceptable strategy for BMT patients, in whom the risk for infections and morbidity are high, it would be of little use in patients with hematological malignancy not on active treatment, in whom the risk and morbidity of infection would be low. In BMT patients, it is currently advised to start acyclovir prophylaxis at the start of conditioning therapy and continue until engraftment occurs, resolution of mucositis, or approximately 30 days after HCT [28].

Table 51.3 Treatment of herpes simplex virus infection with acyclovir

	Viral and clinical response					
Parameter	Acyclovir [30] (IV)	Placebo (IV)	p^a	Acyclovir [29] (PO)	Placebo (PO)	p^a
Median day (range) to						
Negative cultures	3 (1–8)	18 (4–49[b])	0.00005	2	>9	0.0008
First decrease in pain	6 (1–10)	14 (1–14[b])	0.05	3	16	0.04
Cessation of pain	10 (7–21)	16 (7–49[b])	0.03	6	16	0.05
Crusting of all external lesions	7 (2–28[b])	14 (6–49[b])	0.01	6	11	0.01
Total healing	14 (6–28[b])	28 (10–49[b])	0.03	8	21	0.01

[a]Compared by Kaplan–Meier plots and analyzed by Mantel–Cox test
[b]Patient removed from study and given open-label acyclovir

The issue of the emergence of acyclovir resistance is an important consideration in this regard. In BMT patients prophylaxis has been associated with a very low risk for drug resistance [38, 39]. In contrast, repeated treatment episodes of established HSV infection in BMT patients have been associated with progressively increasing rates of resistance [40]. An explanation for this apparent lower risk of resistance seen with prophylaxis has been suggested [41]. Certainly, the gradually improving host immunity that occurs with successful marrow transplantation and the relatively short duration of prophylaxis are contributory, because prolonged acyclovir prophylaxis in advanced AIDS patients, who have relentlessly deteriorating immunity, has been associated with a substantial risk for resistance. Initial reports of acyclovir-resistant HSV infection suggested that virulence had been attenuated and the clinical course was milder [42, 43]. However, reports of resistant isolates causing severe clinical illness have also appeared [44, 45].

For the most part acyclovir resistance has been mediated by alterations in the viral-encoded thymidine kinase production [42, 43, 46]. However, mutations in the DNA polymerase and altered substrate specificity for thymidine kinase are other potential modes of resistance [46]. Risk factors for the emergence of acyclovir-resistant HSV infections include severe GvHD and the lack of ganciclovir prophylaxis [47].

For patients with acyclovir-resistant HSV, treatment with foscarnet, a pyrophosphate analogue that directly inhibits viral DNA polymerase (and does not require thymidine kinase for its activity), may be effective [48]. In a randomized trial that compared foscarnet with vidarabine for acyclovir-resistant HSV infections in AIDS patients, foscarnet was both more effective and less toxic [49]. Another alternative is cidofovir. For patients with mild infection, another alternative approach would be to cease antiviral therapy to allow unassisted resolution. If progressive illness should ensue, then antiviral treatment could be initiated. Currently, there are no vaccines for either HSV-1 or HSV-2.

Cytomegalovirus

Cytomegalovirus infections are common in the general population, with serologic surveys demonstrating latent infection in 40–60% of adults in most industrialized countries. Primary infection is often inapparent, but CMV can be a major cause of congenital malformations and hearing loss, mononucleosis, and sexually transmitted disease. Asymptomatic shedding in saliva and genital secretions is occasionally noted; clinical illness is uncommon unless the patient is immunocompromised.

Different strains of CMV have been identified by using two viral envelope glycoprotein genotypes (gB and gH). Some data suggest that infections by gB type 1 strains may be associated with greater survival than other gB types [50]. In another study, life-threatening myelosuppression was noted with gB3 and gB4 isolates, in contrast to gB1 and gB2 isolates [51]. Further studies are needed to ascertain whether characterization of strains may provide insights as to viral pathogenicity and prognostic information as to outcome from infection [52].

Patterns of Infection

Cytomegalovirus can be reactivated from endogenous latent virus or alternatively can be acquired from transfusion of blood products or from an organ graft. Leukocytes are a reservoir of latent virus; the risk for transmission can be reduced by depletion of leukocytes in blood products [53, 54] and avoidance of granulocyte transfusions [55]. Among seropositive and seronegative BMT recipients, the incidence of CMV infection was similar prior to the routine use of CMV seronegative blood product support [56]. Now infection rates are less than 10–15% in seronegative recipients given seronegative blood products (although higher in the recipients of seropositive marrow grafts) [57].

In immunocompromised patients, CMV can be a cause of fever, leukopenia, thrombocytopenia, esophagitis, enterocolitis, interstitial pneumonitis, hepatitis, mononucleosis-like syndrome, chorioretinitis, and rarely, meningoencephalitis. Infection with CMV may predispose immunocompromised patients to sepsis [58, 59].

Although CMV excretion is a frequent occurrence in patients with acute leukemia undergoing intensive chemotherapy, its relationship to morbidity is poorly defined. However, several small surveys of acute leukemia patients suggest that the morbidity attributable to CMV may be underestimated [60–62]. Among leukemia patients, gastritis and esophagitis occasionally occur, and rarely, pneumonitis can be noted. In contrast, CMV can be the cause of life-threatening illness in BMT recipients.

The most common severe manifestations of CMV in BMT patients are interstitial pneumonitis (Fig. 51.1) and enterocolitis. Less serious manifestations are unexplained fever, esophagitis, gastritis, wasting, and hepatitis. Chorioretinitis is uncommon (in contrast to HIV-infected patients) [56]. Myelosuppression, manifest as delay in the recovery of counts after chemotherapy or unexplained cytopenias weeks to months following engraftment, can occur and can pose diagnostic challenges to distinguish from other causes of myelosuppression.

As with HSV, anti-CMV IgG antibody develops after primary infection. It provides a marker of endogenous latent virus. It does not appear that CMV-specific antibody protects against severe disease because several studies have not found a protective effect of high pretransplant antibody levels against infection [63]. Likewise, there is poor correlation between antibody responses and clinical responses [63]. Thus, while

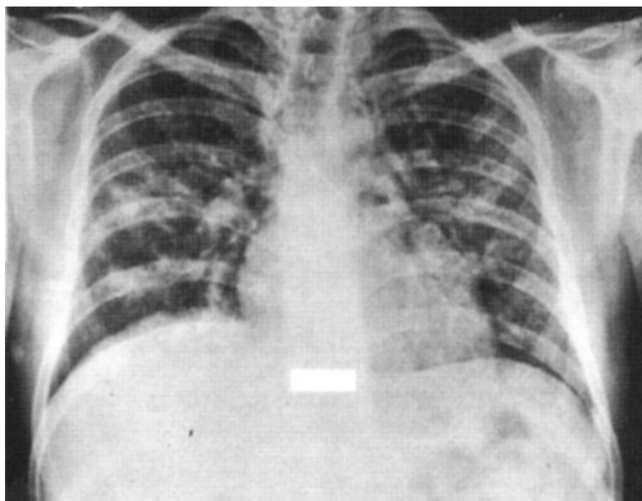

Fig. 51.1 Chest radiograph showing a diffuse mixed alveolar/interstitial pneumonitis due to cytomegalovirus

CMV antibody may have a contributory role in the control of CMV infection, it does not appear to be the most important response. Lymphocytopenia has been noted to be an unfavorable prognostic factor in BMT patients with CMV infection [64], and CMV-specific T-cell and natural killer (NK) responses have been noted following CMV infections [65–68]. The majority of survivors of CMV infection in BMT recipients develop either NK or T-cell cytotoxic responses or both, whereas few of those who succumb from infection develop cytotoxic responses and in the minority in whom cytotoxic responses could be found, the magnitude was much lower [65]. Thus, it appears that the early development of a robust cytotoxic response is crucial to resolution of CMV infection.

Although the development of cytotoxic responses is clearly immunoprotective, animal studies suggest that some cellular responses may be contributory to the pathogenesis of CMV pneumonitis [69–73]. Elucidation of which host immune responses are immunopathogenic and which are immunoprotective is crucial to the understanding of CMV pathogenesis and may permit insights into ways in which augmentation of certain responses and abrogation of others may be possible in future engineering of the constituents of the marrow graft through cell sorting techniques that discriminate various cell populations by their immunophenotypic differences.

Allogeneic BMT recipients are at are at greatest risk for serious illness from CMV infection, in contrast to autologous or syngeneic transplant recipients or nontransplant leukemia patients [74–79]. Among allogeneic recipients, risk factors for CMV-related morbidity and mortality have included HLA disparity between donor and recipient, unrelated donor transplant, T-cell depletion, pretransplant CMV seropositivity, GvHD, older age, the use of immunosuppressive agents other than cyclosporine as GvH prophylaxis, viral excretion, and the intensity of the cytoreductive preparative regimens [20, 56, 57, 75, 79–85]. Detection of viral excretion from blood, urine, or throat secretions is indicative of active infection, and isolation of virus often precedes CMV disease by several days to 1–2 weeks. Viremia is most predictive of subsequent CMV disease [84]. Alternatively, recovery of virus from bronchoalveolar lavage specimens even in patients without any pulmonary symptomatology also identifies patients at high risk for the development of subsequent CMV pneumonitis [86, 87]. In a recent retrospective review of 421 cases that occurred between 1986 and 2011 at the Fred Hutchinson Cancer Research Center, Erard et al. found that female sex, increased bilirubin, lymphocytopenia, mechanical ventilation, and development of grade 3 or 4 graft-versus-host disease increased the risk of mortality from CMV pneumonia as did the lack of treatment with antiviral therapy. In an analysis of 233 cases that occurred in the era of preemptive therapy, only the presence of lymphocytopenia was found to increase the risk of morality from CMV pneumonia [88].

The CMV serostatus of the allogeneic BMT recipient is a significant factor in the long-term outcome. Positive CMV serostatus of the recipient remains a poor prognostic factor, especially in recipients of T-cell-depleted marrow or stem cells. An association of CMV with GVHD and nonviral infections or sepsis has been suggested as a possible mechanism [89]. A large study from the European Bone Marrow Transplant Registry demonstrated the importance of donor serostatus among CMV-seropositive recipients of unrelated grafts, possibly as a result of transferred CMV-specific immunity from the donor to the recipient [90]. In an analysis of a large cohort of T-cell–replete SCT recipients, both donor-positive/recipient-positive and donor-positive/recipient-negative recipients had a higher risk of mortality [91]. After controlling for neutropenia and CMV disease, only donor-positive/recipient-negative recipients had a higher risk of mortality. This was attributed to indirect immunomodulatory effects of CMV because there was an excess mortality due to bacterial and fungal infections when compared with donor-negative/recipient-negative recipients [91]. More recent review of data from 9469 allogeneic transplant recipients transplanted between 2003 and 2010 reported to the Center for International Bone Marrow Transplant Research (CIBMTR) show an increase in nonrelapse-related mortality for all serology groups (donor positive/recipient positive, donor positive/recipient negative, donor negative/recipient positive) compared with the donor negative/recipient negative cohort [92]. Collectively, these studies of the effect of CMV serostatus suggest that CMV infection before transplantation remains an important factor leading to poor outcome after transplantation, especially in recipients of T-cell-depleted transplants.

Although autologous BMT patients are at lower risk for CMV disease, such disease occasionally occurs. Patients

receiving transplants for hematologic malignancy are at greater risk than those receiving transplants for solid tumors. Patients with multiple myeloma undergoing stem cell transplants in one study were at greater risk for CMV disease [93]. In one study, CD34 selection in autologous stem cell transplant recipients was associated with an increased risk for CMV disease [94]. However, this has not been observed by other groups [95].

Of interest, a number of studies have previously evaluated the possible association of early reactivation of cytomegalovirus after allogeneic stem cell transplantation for acute myelogenous leukemia and a decreased incidence of relapse [96]. One study even suggested a similar positive benefit in patients transplanted for chronic myelogenous leukemia. This reduction in incidence in relapse was also suggested to improve overall survival in this group of patients. Unfortunately not all studies have found a positive benefit of CMV reactivation in regard to relapse and/or overall survival [97]. Moreover, an analysis by the European Society for Blood and Marrow Transplantation even suggesting a higher risk of leukemia relapse with a decrease in overall survival in patients who were seropositive for CMV at the time of allogeneic transplantation [98]. With these conflicting findings, the Center for International Blood and Marrow Transplant Research (CIBMTR) recently reported results of analysis of data from 9469 patients transplanted with bone marrow or peripheral blood between 2003 and 2010. Patients were categorized by underlying disease at the time of transplant (AML, ALL, CML, and MDS). The median time to initial CMV reactivation was 41 days. There was no evidence of decrease risk of relapse regardless of underlying disease type in this study. In addition, there was an increase risk of nonrelapse-related mortality. The conclusion of the authors after analyzing this large data set was that CMV reactivation did appear to confer protection against relapse of hematologic disease and remained a risk factor for poor overall outcome [99].

Diagnosis, Prophylaxis, and Treatment

Historically, infection with cytomegalovirus was diagnosed by the use of cultural methods [100]. Unfortunately, this method was not very useful in timely treatment decisions because results may take several weeks. Modified cultural techniques using the shell vial method coupled with a rapid diagnostic antigen assay for the immediate early antigen were then developed to permit demonstration of the virus within several days [101–103].

Newer assays, including the demonstration of the pp65 antigen in circulating leukocytes using immunofluorescence [104–106] or of viral DNA by the PCR [107–110], have replaced the cultural methods of monitoring for active cytomegalovirus infection. Both the leukocyte antigen detection and PCR assays permit detection of virus 7–10 days earlier than shell vial culture. Detection of virus DNA in plasma by PCR appears to be more sensitive than detection in buffy-coat leukocytes. Both the leukocyte antigen assay and PCR assays have the advantage of permitting quantification. Larger virus quantity may be more predictive of subsequent disease than less virus [111]. In one comparison, PCR appeared to be slightly more sensitive than the leukocyte antigen detection assay [112].

Table 51.4 Interstitial pneumonia. Diagnostic procedures

Procedure	Yield (%)	Morbidity (%)
Transtracheal aspirate	5–10	2–5
Bronchoscopy		
Biopsy alone	30–55	5–20
Bronchoalveolar lavage	40–90[a]	5–20
Transthoracic needle		
Aspirate	17–60	10–30
Biopsy	20–60	10–60
Open biopsy	100	5–10[b]

[a]Results include histology, immunofluorescence, and in situ hybridization
[b]100% incidence of pneumothorax requiring chest tube placement

The gold standard for documenting CMV pneumonia in the past had been an open lung biopsy (Table 51.4). Because only half of the episodes of interstitial pneumonitis after marrow transplantation are due to CMV and the treatments are toxic, it is important to confirm the etiology at the outset. Similarly, for gastrointestinal syndromes attributable to CMV, tissue documentation is important to differentiate CMV from GvHD or other etiologies [113]. In contrast to patients with HIV infection, in whom CMV is frequently a pulmonary copathogen of less clinical importance, in BMT patients CMV typically can cause severe illness as a single pathogen. Transbronchial biopsy has not proved to be very reliable, yielding frequent false negatives. In contrast, bronchoalveolar lavage has been found to have a high sensitivity and specificity for documentation of CMV as an etiology of interstitial pneumonitis and is the usual method of diagnosis of CMV pneumonitis today [114]. This less invasive procedure has encouraged clinicians to be more aggressive in the evaluation of patients suspected of having pneumonitis and permits an earlier initiation of therapy.

Untreated CMV pneumonitis after marrow transplantation has an 80–90% mortality. A number of immune modulators and antiviral agents alone and in combination had been tested without success, including vidarabine, interferon, vidarabine plus interferon, acyclovir, acyclovir plus interferon, CMV immunoglobulin, ganciclovir, and ganciclovir with corticosteroids [115–119].

Ganciclovir, a nucleoside analogue similar in structure to acyclovir, differs from acyclovir in that it does not require phosphorylation by a viral-encoded thymidine kinase. Because human CMV does not encode for this enzyme, acyclovir has much less activity than ganciclovir against CMV. Phosphorylation of ganciclovir occurs by the phos-

photransferase product of the viral UL97 gene. Although ganciclovir was shown to have an antiviral effect in BMT patients, most patients initially treated with this agent aloe were felt to have derived little clinical benefit [118]. Several nonrandomized trials which evaluated treatment of CMV pneumonia using a combination of ganciclovir combined with the infusion of intravenous immunoglobulin were felt to show an increase in clinical benefit, with survival rates in the range of 50–70% [120–123]. Although no controlled trials have been performed, this combination has been widely adopted for the treatment of CMV pneumonitis [124]. In a recent review by Erard et al. of 421 cases treated at the Fred Hutchinson Cancer Center from 1986 through 2010, the authors felt their date documented the improvement in outcome of patients with cytomegalovirus pneumonia over the last few decades and was clearly attributed to the availability of specific anti-CMV antiviral therapy. However, the analysis of their data failed to show benefit of the addition of either intravenous pooled or CMV-specific immunoglobulin. If, however, therapy is delayed until ventilatory failure has ensued, outcomes are uniformly poor [88]. Thus, early intervention is crucial. Following control of CMV pneumonia with ganciclovir and immune globulin, there can be occasional recurrences, and some clinicians advocate a maintenance course of ganciclovir for several weeks to several months following an episode of CMV pneumonia.

Although a small prospective randomized clinical trial failed to show statically significant benefit in the use of antiviral therapy with ganciclovir in the treatment of CMV-associated enteritis in marrow transplant recipients in regard to clinical symptoms and endoscopic appearance, the study did show reduction in both orophyngeal and urinary excretion of virus. Four patients on the treatment arm of this study and six on the placebo arm developed pneumonia. All ten of these patients died [125]. Of note, there was less than 20 patients on each arm of the study and it was conducted over 20 years ago before the availability of more sensitive techniques for early viral detection (i.e., detection of CMV viremia in the blood by antigen assay or PCR) as well as the advent of preemptive anti-CMV antiviral therapy as is discussed below. It has, therefore, become standard of practice to treat with appropriate antiviral therapy when CMV enteritis is documented by biopsy. In a subsequent retrospective study by the European Group for Blood and Marrow Transplantation, there appeared to be no improvement in outcome with the addition of intravenous immunoglobulin to antiviral chemotherapy in patients with CMV enteritis [126].

Prior to the availability of effective antiviral therapy for CMV, the onset of CMV-related pneumonitis in the marrow transplant occurred approximately 2 months following transplantation. This was usually shortly after engraftment and frequently after the appearance of acute GvHD. Despite the gratifying improvements in the treatment of CMV pneumonia, many patients still died due to the development of this complication.

Table 51.5 Strategies to cytomegalovirus (CMV) disease in bone marrow transplant patients

Intervention	References
CMV-negative blood products	[50, 82, 115, 131, 132]
Leukocyte filters	[47, 116]
Immunoglobulin or plasma	[117–123]
Acyclovir	[124, 125]
Ganciclovir	[126–129]

A number of investigators have sought ways to prevent either infection or illness from infection (Table 51.5). For CMV seronegative recipients whose donors are also seronegative, the use of CMV-screened blood products has been highly successful in minimizing the risks for CMV infection and disease [57, 104, 127–129]. Alternatively, the use of second-generation leukocyte filters for blood product transfusions similarly reduced the risk and now has become standard of practice in most centers treating patients with hematological malignancies in the United States and Europe [54, 130].

Maribavir has been explored for the prevention of CMV infection in allogeneic stem cell transplant recipients. Despite encouraging results in pilot studies, a subsequent randomized trial showed no significant benefit with maribavir prophylaxis [133].

At present there are no satisfactory preventive measures for seronegative recipients who receive grafts from seropositive donors. Although immune globulin would be appealing in this situation, unfortunately it has not proved to be effective in reducing infection rates [127]. Prophylaxis with acyclovir or valcyclovir in combination with monitoring for viremia and treating viremia with preemptive therapy is advised by recently published consensus guidelines in this population [134].

As noted above, although some centers utilize a prophylactic approach to prevention of CMV disease in their transplant patients, due to the toxicity of the available agents, the "preemptive" approach is most common. The choices of antiviral therapy for the treatment or prophylaxis of CMV disease with several agents. There is clearly an unmet need for antiviral agents with less toxicity as well as enhanced antiviral activity against cytomegalovirus isolates that shows resistance to the currently available agents. Some of the antiviral agents with activity against CMV that show promise and are currently under study include maribavir, letermovir, brincidofovir, and leflunomide [135].

Maribavir, a UL97 protein kinase inhibitor is an orally bioavailable drug with activity agents CMV. In a recent phase III clinical trial utilizing this drug as prophylaxis for CMV infection in allogeneic stem cell transplant recipients,

mirabivir failed to show benefit in prevention of CMV disease. A prior phase II dose range study in hematopoietic stem cell transplant patients had shown activity of the drug against CMV infection or disease at all three different dose levels tested. Since the phase III clinical trial utilized the lowest dose of drug study in the phase II trial (100 mg twice daily), failure of maribavir to prevent CMV disease in the prophylaxis study was considered to be due to suboptimal dosing of drug. Maribavir has also shown activity against CMV resistant to ganciclovir or cidofovir in vitro with small case series suggesting a possible clinical. Further dose ranging studies are currently ongoing utilizing maribavir as preemptive, rather than prophylactic therapy and in transplant recipients with CMV infections that are refractory or resistant to prior antiviral therapy.

Letermovir is a CMV terminase inhibitor with a high level of activity against wild type and drug resistant CMV. Being specific for CMV terminase, it has little activity against other members of the herpes group viruses. The drug is available in both intravenous and oral form and was shown to be superior to placebo at a dose of 240 mg in a phase II dose-escalation study when used as prophylaxis in a group of HLA-matched transplant recipients who were CMV seropositive. As a follow-up of the phase II study, a phase III randomized clinical trial is ongoing. Letermovir has been found to be well tolerated and has been given fast track status by the US Food and Drug Administration and orphan drug status by the European Medicines Agency [136].

Unlike letermovir, brincidofovir is a new antiviral agent with a broad spectrum of activity against herpesviruses other than just CMV, polyomaviruses, adenoviruses, papillomaviruses, and variola virus. It is a lipid analogue of cidofovir which highly bioavailable orally with a long half life. The drug has much less renal toxicity as it is not a substrate for human organic anion transporters. Used a prophylaxis starting at the time of engraftment after allogeneic hematopoietic stem cell transplantation, brincidofovir showed a reduction in CMV infection or disease at a dose of 200 mg per week. Most common side effect was diarrhea. There was no evidence of increased renal or hematological adverse events compared to placebo. A phase III randomized trial for prophylaxis in hematopoietic transplant patients is ongoing [137].

Other drugs which have been studied for the treatment of CMV infection and disease include leflunomide which is FDA approved for the treatment of arthritis. This drug has been shown to have activity against CMV and BK virus. Although it has been reported as being used as a salvage agent for CMV disease with mixed results, no well-controlled studies with this agent for the treatment or prevention of CMV disease have been conducted.

The antimalarial drug arteunate has also been noted to have broad activity against herpes group viruses as well as hepatitis viruses and human immunodeficiency virus due to its ability to downregulate NF-kappB or SP1 pathways. Despite a few reports of efficacy in patients with multi-resistant CMV disease, no formal studies of efficacy and/or toxicity have been performed with this drug [135].

Immune globulin, either CMV specific or from standard commercial lots, has also been shown to be beneficial in reducing CMV infection or disease [138–141]. It is not clear whether there is any advantage to using a hyperimmune globulin preparation compared to commercial lots of immune globulin not specifically selected for high titer anti-CMV antibody. Most of the efficacy from immune globulin has been demonstrated in patients who were seronegative prior to transplant. There does not appear to be any additive benefit of immune globulin to the use of filtered blood products in seronegative patients [126]. The magnitude of benefit from immune globulin has been less in seropositive patients [142], and immune globulin has been found to be of no benefit in autologous marrow transplant recipients [143].

Before the availability of antiviral agents with greater activity against cytomegalovirus, such as ganciclovir and foscarnet, attempts at infection prevention with high-dose intravenous acyclovir were studied. In one such study, seropositive patients receiving high-dose intravenous acyclovir given during the first month after transplantation suggested there was a reduced risk for CMV pneumonia [144]. In a three-arm trial, high-dose intravenous acyclovir during the first month, low-dose oral acyclovir during the first month, and high-dose intravenous acyclovir during the first month followed by 6 months of oral acyclovir were compared [145]. A reduced probability and delayed onset of CMV infection was noted in the group receiving high-dose intravenous acyclovir [145]. In this latter study, the prolonged course of oral acyclovir did not reduce CMV infection but did reduce viremia, and there was an improved survival benefit [145]. Similar acyclovir prophylaxis trials in solid organ transplant recipients had shown efficacy. Why acyclovir as prophylaxis seems to exert an anti-CMV effect whereas its use as treatment provides no effect is puzzling but may be indicative of a mild antiviral effect, which is demonstrable only when the viral burden is low as in the early stages of active infection and not apparent in later stages of infection, when the viral burden is substantially greater.

With the availability of an antiviral agent with greater activity in the early 1990s, however, ganciclovir was also evaluated for prophylaxis (from engraftment to day 100) or as early treatment in BMT patients with active virus infection to preempt disease. As noted previously, asymptomatic patients with virus recovered from bronchoalveolar lavage fluid 35 days after transplantation, as well as those in whom viremia or excretion in urine or throat specimens occurs are at higher risk for CMV disease. Initial studies using ganciclovir as prophylaxis in seropositive patients significantly reduced the incidence of life-threatening CMV disease; how-

ever, the incidence of cytopenias, particularly neutropenia, increased the risk of bacterial and fungal infections in patients receiving this antiviral agent versus placebo controls negated much of the benefit in regard to overall outcome. An alternative approach has been to use viral detection methods (antigenemia assay or DNA titer by PCR) of serial blood samples to detect development of viremia and then "preemptively" start antiviral therapy in attempt to avoid significant marrow suppression with this agent before the development of end organ disease (mainly viral pneumonia). This has become the favored approach in many transplant centers today. An alternative prophylactic strategy is to initiate ganciclovir at the time of engraftment in all seropositive patients [146, 147]. Because of its myelosuppressive toxicity, it is difficult to begin the use of ganciclovir as prophylaxis prior to engraftment. Even when it is begun shortly after engraftment, pancytopenia and interruptions of treatment are common, with an attendant risk of neutropenic infections. Patients with low marrow cellularity, elevated bilirubin, or increased creatinine are at greater risk [148]. The most common practice at present is viral surveillance utilizing polymerase chain reaction with preemptive initiation of antiviral therapy when CMV reactivation is documented. This practice has reduced the risk of death from CMV after allogeneic stem cell transplantation to less than 10% [56].

The use of myeloid growth factors can lower the risk of myelosuppression. There is divided opinion at present as to the relative merits of prophylaxis versus early "preemptive" therapy [39, 149]. Several reviews have discussed the issues related to these strategies [39, 150–153]. It appears that prophylaxis is associated with fewer failures that could occur before or simultaneously with the first detection of active virus. However, the greater morbidity attendant on more frequent and more prolonged pancytopenia with ganciclovir started at the time of engraftment makes ganciclovir less well tolerated than when started later. Moreover, early therapy given only to patients with active infection spares a significant proportion of patients the toxicities and cost of antiviral therapy. The greater use of the leukocyte antigen and PCR assays to detect virus earlier than with the shell vial culture assay may reduce some of the failures associated with early therapy, making this more advantageous than prophylaxis [39]. If ganciclovir is used for prophylaxis or as early therapy, there appears to be no added benefit for acyclovir during the first month [154].

In an attempt to reduce the toxicity of long-term ganciclovir, several variations of daily therapy have been attempted. Ganciclovir three times per week was not found to be adequate to prevent CMV reactivation, at least in T-cell-depleted marrow transplant recipients [155]. On the other hand, a preliminary report suggests that 3–6 weeks of daily ganciclovir may be as efficacious as a longer course while causing less toxicity [156, 157].

Oral ganciclovir is useful in AIDS patients for maintenance therapy to prevent recurrence of CMV retinitis, but because of poor bioavailability, serum concentrations are lower and breakthrough infection rates appear to be higher [158]. Emergence of resistance has also been noted and appears to be greater than with intravenous ganciclovir [159, 160]. Valganciclovir, a prodrug of ganciclovir, is metabolized to ganciclovir and has excellent bioavailability [161–163]. Plasma concentrations are quite high. Valganciclovir has been shown to be an alternative to ganciclovir, which may make prolonged courses of therapy more feasible [164–166].

Resistance to ganciclovir occurs through mutations in the CMV UL97 gene [167–169]. DNA polymerase (UL54) mutations can also occur, resulting in high-level resistance [169]. Although many ganciclovir-resistant CMV mutants have mutations in the UL54 gene (DNA polymerase) as well as in the UL97 gene, susceptibility to foscarnet is retained [169–171]. Although rising CMV antigen titers can be seen in approximately 40% of patients early on with ganciclovir maintenance therapy, most isolates are susceptible to ganciclovir, indicating that host factors and medications, rather than resistance, accounts for the rise in titers. Additionally, no correlation was seen between CMV titers and CMV disease [172].

Foscarnet, a pyrophosphate analogue, targets the viral DNA polymerase. It represents an alternative to ganciclovir both as treatment and as prophylaxis [173–177]. Sparing of the blood counts makes foscarnet an attractive alternative to ganciclovir in patients in whom marrow reserve is marginal. However, nephrotoxicity and renal wasting of electrolytes are prominent side effects of foscarnet. In one small study, the combination of foscarnet and ganciclovir was effective and may have less treatment toxicity [178]. Foscarnet is an option for ganciclovir-resistant CMV [179].

Cidofovir is a nucleoside analogue with potent anti-CMV activity not requiring phosphorylation by the UL97 gene product, which acts against the viral DNA polymerase (UL54). It is an effective treatment for CMV retinitis in HIV-infected patients [180–183]. Advantages include less frequent dose intervals (once weekly at first, then every other week). Limitations include considerable nephrotoxicity (which in some cases was irreversible) and iritis. Concomitant probenecid and intravenous hydration are necessary. There are to date only limited data on BMT patients [171]. Because many ganciclovir-resistant CMV mutants have alterations not only in the UL97 gene but also in the UL54 gene (DNA polymerase), resistance to cidofovir can also be present [169, 170]. Accordingly, foscarnet is the preferred antiviral in the face of ganciclovir resistance.

Historically, most episodes of CMV pneumonitis occurred during the first 100 days after marrow transplantation. However, with the advent of antiviral prophylaxis in recent

years, late-onset CMV pneumonitis has increasingly been noted beyond 100 days [184–190]. In part this is due to the increasing use of unrelated and HLA-mismatched donors, where immune reconstitution is much slower than after allogeneic transplants from genotypically identical siblings. However, delay in the development of cytotoxic anti-CMV responses in patients in whom ganciclovir has been used has also been noted [191, 192]. Most late-onset cases occur in patients in whom CMV infection was documented during the first 100 days [186]. The risk of late-onset CMV pneumonitis appears to be greatest in patients with chronic GVHD. This is a group in whom CMV surveillance may be particularly important. With recognition of the importance of development of cytotoxic cellular responses to CMV for resolution of infection as well as of the ability to clone lymphocyte populations and expand them ex vivo with interleukin-2 and repetitive CMV antigenic stimulation, it has become possible to consider cellular immunotherapy of CMV infection [193–197]. Consideration of donor immunization may also be important because recipients of grafts from seropositive donors may have earlier recovery of cytotoxic responses than recipients of grafts from seronegative donors [191]. Such immunotherapeutic approaches may prove to be increasingly important in years to come as the emergence of ganciclovir resistance among CMV isolates increases [198].

Because the risk of CMV disease is substantially greater in seropositive than in seronegative patients, it has been previously recommended that newly diagnosed leukemia patients who are seronegative for CMV and in whom a transplant is contemplated after induction therapy should receive only CMV-seronegative blood products throughout their induction therapy. The benefit of the routine use of this practice is questionable so long as only leukopoor filtered products are given, particularly if CMV seronegative blood is not available. The value of CMV-negative blood products in autologous transplantation patients, in whom the risk for serious CMV disease is low, is questionable but has been recommended by some [199] This practice, however, has been mostly abandoned with the routine use of leukocyte filtered blood products for transfusion support. Although the use of seronegative blood products and/or routine leukofiltration of donor blood has markedly reduced the risk of transfer of CMV to recipients, newer methods of pathogen inactivation of donor may also reduce the risk of viral transmission to recipient in the near future [200].

Immunization of the donor or recipient by a live attenuated, killed, or subunit recombinant CMV vaccine might be attractive in speeding restoration of immunity [201]. However, although an attenuated vaccine has been found to be immunogenic in solid organ transplant patients, concerns regarding its safety in more severely immunodeficient marrow transplant patients have prevented trials of a live vaccine. A glycoprotein or DNA vaccine would be desirable. Clearly, abrogation of the recipient's immunity by the preparative regimen may mean that prior immunization of the donor as well as repeated boosting of the recipient is necessary to establish meaningful immune responses..

Varicella Zoster Virus

Patterns of Infection

Varicella zoster virus, like the other herpesviruses, can be a significant pathogen in patients with hematologic malignancies. Varicella (chickenpox) is the primary form of infection, and zoster (shingles) is the reactivation form of illness. The initial portal of entry is uncertain, but the respiratory tract, skin, and conjunctiva are candidate sites. After local replication, viremia occurs with dissemination to multiple cutaneous (and visceral) sites. The skin lesions begin as erythematous macules, progress to papules and then to vesicles (with clear fluid), which evolve to pustules (with cloudy fluid containing interferon and leukocytes), and flatten to scabs, until finally healing takes place. The hallmark of infection is the vesicle. Crops of vesicles classically exist on a common erythematous base. Scarring is unusual in varicella, but the inflammatory reaction in zoster may be more severe with greater pain and scarring. Zoster characteristically is dermatomal, and lesions typically stop at the midline. In severely immunocompromised patients extension to adjacent dermatomes may occur, the vesicles may become confluent, and cutaneous dissemination can occasionally occur. Approximately 5–10% of patients with non-Hodgkin's lymphoma, 15–25% of patients with Hodgkin's disease, and 20–50% of patients undergoing BMT develop zoster [202–207] (Table 51.2).

In the normal host varicella and zoster are self-limited. In patients with hematologic malignancies, varicella may be quite severe and life threatening. In the preantiviral era, varicella frequently progressed to visceral dissemination and death [208].

Untreated dissemination occurs in 30–40% of marrow transplant recipients [204, 205] and in up to 20–30% of children treated for hematological malignancy [208]. Visceral dissemination is a severe manifestation and pneumonia can be particularly deadly. In marrow transplant patients an intra-abdominal presentation can occur, with severe abdominal pain [209–211]. This can be particularly challenging to diagnose because it may occur in the absence of cutaneous vesicles. A high index of suspicion should be maintained because if the condition is not treated promptly, it can result in a high mortality. Manifestations of pancreatitis, hepatitis, and peritonitis are frequent, and in patients in whom laparotomy was inadvertently performed, inflammatory changes, vesicles on serosal surfaces, and mesenteric adenitis have been noted.

The onset of VZV infection in the marrow transplant recipient is much later than that of the other herpesviruses,

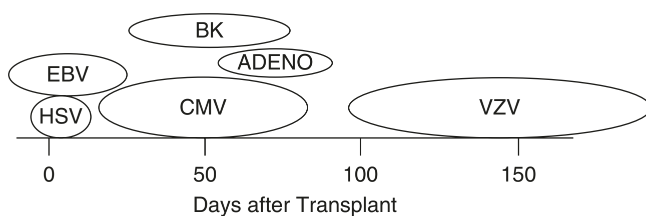

Fig. 51.2 Viral infections and their time course after marrow transplantation. *HSV* herpes simplex virus, *EBV* Epstein–Barr virus, *CMV* cytomegalovirus, *BK* BK virus, *ADENO* adenovirus, *VZV* varicella zoster virus

occurring an average of 5 months following transplantation [204–207] (Fig. 51.2). Susceptibility for reactivation can persist for many months, especially among patients with chronic GvHD [204, 205]. A study of humoral and lymphocytic proliferative responses to VZV in BMT recipients indicated that in approximately 26% of patients a subclinical reactivation occurred [212]. Similarly, in patients undergoing induction therapy for leukemia, 5–10% subclinical reactivation occurred. Thus, when both clinical and subclinical infections are considered, VZV infection is a common occurrence in BMT recipients.

Among patients treated for lymphoma, VZV infection is more common in those with advanced disease, those treated with combined-modality chemoradiotherapy [202], and in patients who have received rituximab [213]. It appears that the frequency of zoster is increasing as more dose intensive therapies are being employed. Risk factors for VZV in allogeneic marrow transplant patients include acute or chronic GvHD, older age, and the posttransplant use of antithymocyte globulin. Among autologous transplant patients, the underlying disease of Hodgkin's or non-Hodgkin's lymphoma is a risk factor [206]. Zoster develops more commonly in dermatomes of prior radiotherapy.

Diagnosis and Treatment

Diagnosis can usually be made solely by visual inspection of the cutaneous lesions. Examination of the cellular contents of a vesicle can demonstrate multinucleated giant cells on Tzanck smear [25]. Culture can be confirmatory but special handling must be used for this heat-labile cell-associated virus. Immunofluorescent staining can also be used [214]. PCR techniques may offer yet another alternative [25].

Acyclovir is highly active against VZV [215–217]. Higher concentrations are required to inhibit VZV in vitro as compared to HSV-1 and HSV-2. Generally, plasma levels achieved with standard doses of acyclovir given orally are insufficient to achieve inhibitory plasma concentrations. High doses of oral acyclovir (800 mg given five times daily) achieve peak plasma concentrations that approximate in vitro inhibitory concentrations for most isolates of VZV and have been found to be useful in nonimmunocompromised patients in whom treatment is initiated early after onset of infection [218]. The adequacy of this dose in compromised patients has not been well demonstrated, although one small trial in BMT recipients suggested that a beneficial effect was achieved [219]. However, because only a small number of patients have been studied, intravenous acyclovir is preferable as initial therapy unless the infection is mild and the patient can be closely monitored to institute parenteral therapy if there is progression or no response.

Vidarabine in the past was an alternative treatment [220], although controlled trials have shown acyclovir to be superior [217]. Interferon-α has also been shown to be efficacious [221], although it has been evaluated only in localized disease and there is no published experience of its use in disseminated disease [222].

Several oral agents are effective therapies in nonimmunocompromised patients. These include famciclovir and valacyclovir, prodrugs of penciclovir and acyclovir, respectively [223–225]. These agents have greater bioavailability and longer half-lives than oral acyclovir, which make them excellent candidates for outpatient treatment. Their efficacy has not been shown to be suitable for zoster therapy, including in immunocompromised patients [226, 227]. They seem to be suitable for mild to moderately severe cases of zoster when the patient can be observed carefully or as a change from intravenous acyclovir once an initial response to therapy is achieved.

The risk for acyclovir resistance in VZV is much less than in HSV. However, several reports have noted the emergence of drug resistance [228, 229]. As with HSV, the mechanism of resistance appears to be an altered thymidine kinase, and thus foscarnet is a suitable alternative for resistant pathogens [229].

Immunization and Prophylaxis

A live attenuated vaccine has been evaluated in normal and immunocompromised children with leukemia and found to be beneficial in reducing the risks for VZV infection in patients with ALL in remission for more than 1 year [230–233]. Because of the risks of dissemination by the vaccine strain in patients with severe immunocompromise, its role in such patients is limited [234]. On the other hand, family members of seronegative children with leukemia could be immunized to reduce the risk of the patient contracting a primary infection [235].

Because of the high mortality of VZV-associated disease among severely immunocompromised HCT recipients and the lack of clinical data, passive immunization with either VZIG or VariZIG is advised under very specific circumstances [134]. For VZV-seronegative immunocompromised HCT recipients, VZIG or VariZIG should be administered within 96 h after close or household contact with a person with chickenpox or shingles. VZV-seropositive patients who

are highly immunocompromised can be given VZIG or VariZIG after exposure to VZV, including after exposure to a VZV vaccine and developing a varicella-type rash. Postexposure acyclovir or valacyclovir may be used as an alternative if VZIG or VariZIG is not available [236, 237].

Acyclovir given orally in intermediate doses for a number of months has been shown to reduce the risks for VZV infection following marrow transplantation [212, 238–242]. Administration of acyclovir for 1 year in BMT recipients has been shown to be effective without a rebound of zoster disease after discontinuation [243]. In one study, the possible transfer of VZV-specific T-cell immunity from donor to recipient in the marrow transplantation setting was evaluated [244].

Unfortunately, simple transfer of immunity was not evident. Whether boosting the donor immunity prior to adoptive transfer might improve on this remains to be seen. Currently, it is advised that autologous and allogeneic transplant recipients receive acyclovir prophylaxis for 1 year after transplantation [134]. In patients who have cGVHD or are on chronic immunosuppression, a longer duration of prophylaxis is required.

Epstein–Barr Virus

Pathogenesis and Immunity

Epstein–Barr virus is an ubiquitous organism with the majority of adults being infected. Transmission is usually via oral secretions. Sites of virus replication include the oropharyngeal epithelium and B lymphocytes, the latter being the major reservoir of endogenous latent virus [245–248]. Primary infection is often subclinical, but in adolescence EBV can cause the syndrome known as infectious mononucleosis. It can also cause acute or protracted fever, malaise, fatigue, autoimmune hemolytic anemia, cytopenias, rash, or adenopathy [248–250].

Rarely, it has been associated with several varied neurologic syndromes. It has been implicated as a factor in the pathogenesis of African Burkitt's lymphoma, central nervous system lymphomas, polyclonal and monoclonal lymphoproliferative disorders in solid organ transplant patients, nasopharyngeal carcinoma, and lymphoproliferative disorders associated with the X-linked lymphoproliferative syndrome; in addition, a possible role in Hodgkin's disease has been proposed [248, 251–254].

Primary infection elicits both humoral and cell-mediated immune responses [248, 255–258]. During the acute illness, the production of heterophile antibody and autoantibodies occur. The heterophile antibody (antibody to sheep erythrocytes not absorbed by guinea pig antigen) is most commonly used to document acute infection. Early after infection, IgM antibody and IgG antiviral capsid antibody (VCA) appear, along with antibodies to the early antigen (EA). IgM antibody disappears generally within 2–4 months, but anti-EA antibody may persist for several months or longer. Anti-EBNA antibody appears late, several months after the acute infection, and persists for life. Host cellular responses include natural killer cells and EBV-specific cytotoxic and suppressor T cells. The atypical lymphocytosis characteristically seen during infectious mononucleosis is primarily made up of the responding T-cells rather than the infected B lymphocytes, and these represent the host attempt to control B-cell lymphoproliferation.

Within the B lymphocyte, the EBV genome persists as episomes or occasionally becomes integrated into the host cellular genome. In latently infected cells EBV gene expression is restricted to nuclear protein genes (EBNA), a membrane protein (LMP), and EBV-encoded RNAs (EBERs), which have roles in maintenance of latency, transformation, and prevention of apoptosis [258]. Although latent EBV infection confers a proliferative advantage to B cells, by itself it does not appear to be sufficient for oncogenesis. Rather, other events involving alterations in oncogenes such as c-myc or N-ras or mutations in tumor suppressor gene such as p53 or RB appear to be necessary for the transition from polyclonal proliferation of B lymphocytes to a monoclonal B-cell malignancy [259, 260].

Diagnosis and Treatment

Culture of EBV is problematic because of the lack of readily available assays in diagnostic virology laboratories. However, in research laboratories EBV can be identified by its ability to transform cord lymphocytes [261]. Today, assays for viral DNA detection are mostly straightforward and monitoring by quantitative PCR is readily available [262, 263].

Epstein–Barr virus can be detected in salivary secretions in 15–20% of the general population [261]. Excretion rates are higher in immunocompromised patients. Patients receiving immunosuppressive therapy have excretion rates of about 35%, patients with malignancies have excretion rates in the range of 40–50%, renal transplant patients 45–60%, leukemia patients 75–100%, and HIV-infected patients 45–70% [264–269]. In adults, most infections occur in previously seropositive patients, whereas in children both primary and reactivated infections occur. The number of EBV genomes detected in peripheral blood leukocytes is increased in immunocompromised patients.

Excretion rates vary considerably according to the intensity of immunosuppressive therapy and the extent of depression of host cell-mediated immunity. Thus, patients with repeated organ graft failure, those who receive multiple immunosuppressive drugs, and those who receive antithymocyte globulin are especially vulnerable for reactivation. Similarly, depending on the immunologic defect, the serologic responses to EBV infection may be more or less pronounced than in immunocompetent individuals. Frequently,

serologic responses are exaggerated [270], whereas the more important cell-mediated immune responses are generally suppressed.

Although EBV shedding may be asymptomatic, several EBV-associated syndromes have been identified in patients with hematologic malignancies. Oral hairy leukoplakia commonly found in AIDS patients is also noted in patients with hematologic malignancies, especially those undergoing marrow transplantation [271–273]. There has been increasing recognition of EBV-associated lymphoproliferative syndromes in transplant recipients [264, 274–276]. Children appear to be at greater risk than adults, and seronegative patients are at substantially greater risk than seropositive patients. The risk also varies according to type of transplant, with heart–lung transplant recipients appearing to be at greater risk than those with renal, liver, or cardiac transplants. The evidence for a role of EBV as a causative agent includes the presence of EBV DNA and EBV-specific proteins within tumors, the presence of activation markers consistent with latent EBV infection (CD23 and adhesion molecules), the association with primary infection, and the analysis of terminal repeats indicating monoclonality. Several types of posttransplant lymphoproliferative disease (PTLD) have been described. A mononucleosis syndrome that is localized and self-limited is the least serious. A more widespread mononucleosis syndrome, which can be progressive, and lymphoma, which can be extranodal involving the gastrointestinal tract, brain, and other organs, can also occur. Several classification schemes have been proposed, including one which incorporated molecular genetic analyses with morphology [259].

In BMT recipients, EBV infection has been characterized both serologically and by EBV detection [277, 278]. Reactivation of EBV in seropositive recipients is frequent and generally occurs 2–4 weeks following transplantation. Clinical manifestations have not been associated with viral reactivation, however.

Infrequently, PTLD has been noted in marrow recipients, generally in the setting of severe GvHD, the use of antithymocyte globulin or anti-T-cell antibody, or after T cell-depleted, cord blood, or haploidentical donor transplantation [279–286]. In a comprehensive review of more than 18,000 patients who had undergone allogeneic BMT, PTLDs were noted with a cumulative incidence of approximately 1% at 10 years [287]. Most cases occurred between 1 and 5 months posttransplant with few cases occurring after 1 year. In a multivariate analysis, lymphoproliferative disorders within 1 year of transplant were associated with unrelated or mismatched donor transplants, T-cell depletion of the donor marrow, the use of either antithymocyte globulin or antiCD3 monoclonal antibody as part of the GvHD prophylaxis regimen, acute GvHD, and conditioning regimens that included radiation. T-cell depletion methods that did not remove B cells (the target cell of EBV infection) were associated with a substantially higher risk than methods that depleted both B and T cells. The only risk factor for late-onset PTLD was the occurrence of chronic GvHD [287].

Although most EBV-associated lymphoproliferative disorders have been described in solid or BMT recipients or in HIV-associated immunodeficiency, the increasing use of potent purine analogues, such as fludarabine or cladribine in combination with corticosteroids, has created a new population of oncology patients with profound cell-mediated immunodeficiency. A recent study of EBV DNA load in leukemia and lymphoma patients receiving fludarabine, cyclophosphamide, and dexamethasone indicated a substantial increase in viral load in some patients [288]. Although no EBV-associated lymphoproliferative disorders have been noted to date, these are cautionary observations, which warrant further study.

Acyclovir is active against EBV both in vitro and clinically [289–292]. Its clinical efficacy in nonimmunocompromised patients with infectious mononucleosis, however, has been only slight [282], which is perhaps related to the initiation of therapy late during active viral replication. However, acyclovir has been useful in the clinical management of oral hairy leukoplakia [293], and viral shedding has decreased in compromised patients [272, 278].

Ganciclovir and interferon have also been noted to be active against EBV [294–299]. The activity of acyclovir in the treatment of EBV lymphoproliferative syndromes has not been particularly salutary, although at least some individual patients with polyclonal disorders may have benefited [252, 274]. However, in a more comprehensive compilation of cases, there did not appear to be any difference in survival of those receiving acyclovir therapy for EBV-associated lymphomas [276]. More important in the overall management is reduction of intensive immunosuppressive therapy, which can have salutary effects early in the evolution of the lymphoproliferative disorders [248, 268]. Once mutations in oncogenes or tumor suppressor genes have taken place, attempts to reduce immunosuppressive therapy may be futile [259], and cytotoxic therapy may be more appropriate. Soluble CR-2 (the EBV receptor of B lymphocytes) has been proposed as a therapeutic agent that can block EBV infection [300].

In solid organ transplant patients, the number of EBV-infected lymphocytes in the peripheral blood has been noted to correlate with the risk for posttransfusion lymphoproliferative disorder (PTLD) [301]. After BMT, high levels of circulating EBV detected by quantitative PCR have similarly been shown to be associated with the subsequent development of PTLD [302–304]. This affords an early opportunity to intervene "preemptively" by reduction in the immunosuppression if possible.

Rituximab, an anti-CD20 monoclonal antibody, has also been used to treat lymphoproliferative disorders either by itself

or in combination with lymphocytes, and in anecdotal reports it has demonstrated efficacy [305–307]. Administration of rituximab can be used preemptively to prevent PTLD in BMT patients with high levels of circulating EBV [308]. In healthy seropositive individuals, the population of EBV-cytotoxic T-cell precursors in the peripheral blood is much higher than populations of cells sensitized to other herpesviruses. Thus, buffy-coat leukocyte infusions or ex vivo expanded EBV-specific cytotoxic lymphocytes can be used as therapeutic modalities for EBV-associated PTLD [309]. Infusions of EBV-specific cytotoxic T lymphocytes have been given to patients with high levels of virally infected lymphocytes at high risk for PTLD, resulting in a reduction of viral load [302, 310–313]. Antiviral agents have not been successful in preventing the development of PTLD [281, 283, 286]. Gene-marked infused cells can persist for as long as 18 months [314]. Such “preemptive” lymphocyte transfusions offer promise for prevention of PTLD in at risk patients.

Human Herpesvirus Types 6, 7, and 8

Human herpesviruses types 6 and 7 have only been recognized in the last decade. However, infection in humans is common [315, 316]. Both have a preference for CD4-positive T lymphocytes but can infect a variety of T- and B-cell lines. Seropositivity is present in 50–90% of normal adults, and infection generally occurs during the first decade of life. Both appear to share clinical manifestations. Although usually subclinical, exanthem subitum, a self-limited, mild, febrile illness, can occur. Viral antigens to HHV-6 have been noted in tissues from lymphoma, but their significance is as yet unknown [317–319].

Reactivation of HHV-6 is frequently detected in the blood of BMT recipients [320–323]. For the most part this has not been associated with clinical manifestations. However, suggestions have been made that rash [322], GvHD [323, 324], and marrow suppression in the posttransplant setting [325–327] may be associated. Other reports have suggested a role for HHV-6 in interstitial pneumonitis [328, 329], encephalitis [330], and lymphocytopenia [331]. The precise role in pathogenicity of this virus has been debated [332–334].

The true impact of antiviral therapy for the treatment of clinical disease due to reactivation or primary infection with HHV6 remains unknown due to the lack of well-controlled, prospective clinical trials. Antiviral agents including ganciclovir and foscarnet appear to be active against HHV-6 in in vitro assays [335] and have been used anecdotally [336–344].

Much less is known regarding human herpesvirus 7. It has been implicated as a possible cofactor in human herpesvirus 6 infections [344] and has been implicated in a case of acute myelitis in an adult BMT recipient [345].

Human herpesvirus type 8 has been associated with several human malignancies, including Kaposi’s sarcoma (giving rise to its other name, Kaposi’s sarcoma-associated herpesvirus), some forms of lymphoma, and multiple myeloma. This virus has been found in saliva, semen, and peripheral blood mononuclear cells and is thought to be transmitted sexually. Tissue samples from most Kaposi’s sarcomas contain gene sequences in common with HHV-8 [346–348]. Serologic prevalence studies have revealed contradictory findings. Certain forms of B-cell lymphomas have also been described, especially AIDS-associated body cavity lymphomas, as containing HHV-8 sequences [349, 350]. It is thought to be the cause of Casteman’s disease [351]. Several laboratories have reported detection of HHV-8 DNA sequences in patients with multiple myeloma [352]; however, other groups have disputed such claims [353, 354].

Intestinal Viruses

Enteric viruses cause sporadic infections in patients with hematologic malignancies, which generally occur in conjunction with outbreaks within a given community. Common pathogens include coxsackievirus, rotavirus, the Norwalk virus, and other caliciviruses and astroviruses. But other viruses, including adenoviruses, present in the wider community may also cause illness. Coxsackie A1 is an enterovirus that has been reported to cause severe diarrhea in marrow transplant recipients [355]. In one series, episodes of gastroenteritis were clustered temporarily and caused high mortality [355]. At autopsy foamy vascularization of the mucosal epithelium was noted, with patchy sloughing of intestinal mucosal epithelium. This outbreak coincided with diarrheal illness in nonimmunocompromised children in the community from whom coxsackievirus was isolated from stool. Isolated cases of disseminated infection from enteroviruses have been reported [356, 357]. Isolation of the virus from stool is confirmatory of the diagnosis. Coxsackievirus infection must be distinguished from GvHD by intestinal biopsy because the manifestations may be quite similar.

Rotavirus is a double-stranded RNA virus and member of the Reoviridae family. Its target tissue is the small intestinal mucosal epithelium. Fecal–oral transmission is the usual mode of contagion. There is considerable seasonal variation of infections, most occurring between January and April in temperate climates. Rotavirus gastroenteritis can vary in severity from mild, short-lived diarrhea to overwhelming and occasionally fatal gastroenteritis. A cluster of cases has been reported in BMT patients [358]. In another study, frequent stool isolation of rotavirus has been found in autologous and allogeneic marrow transplant recipients with diarrhea [359]. At present there is no specific antiviral therapy; electrolyte and fluid replacement are important adjunctive measures. To

date there have not been cases of Norwalk and related viruses reported in patients with hematologic malignancies, probably because of the lack of widely available diagnostic assays. Humoral immunity is felt to be an important host protective response for these viral pathogens. The administration of oral immune globulin or breast milk has been suggested for the treatment of severe rotavirus infection.

Adenovirus

Adenoviruses are double-stranded DNA viruses. Forty-nine antigenic serotypes (divided into six subgroups, A through F, based on their antigenic properties and hemagglutination properties) have been identified, each associated with different clinical syndromes. For example, serotype 3 has been associated with keratoconjunctivitis, serotype 11 with hemorrhagic cystitis [360–362], serotypes 40 and 41 with gastroenteritis, and serotypes 3, 4, and 7 have been documented in epidemics of acute respiratory diseases in military facilities. Serotype 35, although uncommon in the general population is common in immunocompromised patients.

The means of transmission of adenovirus is uncertain but has been reported to occur via direct contact, aerosols, contact with respiratory or fecal secretions, and contaminated water. The virus is capable of at least three types of interactions with cells. These include a lytic infection, a chronic persistent or latent infection, and oncogenic transformation.

Patterns of Infection

Adenovirus is endemic in the pediatric population, with approximately 80% of children between the ages of 1 and 5 years having antibody to one or more serotypes [363, 364]. Adenovirus is a relatively common cause of respiratory tract infections in children less than 5 years of age, causing 2–7% of respiratory tract infections and 5–11% of viral pneumonia and bronchiolitis cases [365]. These infections are rarely severe and typically manifest as upper respiratory tract infections. Other presentations include keratoconjunctivitis, gastroenteritis, cystitis, myocarditis, and lower respiratory tract infections.

Adenovirus infections are well documented in immunocompromised patients, including BMT recipients (4–21%), solid organ transplantation patients (liver transplants 8–18%, renal transplants 12%), and patients with AIDS (28%). The frequency with which adenovirus infection and disease is reported among BMT patients is increasing. This is due in part to increased intensity of immunosuppression and to the use of T-cell-depleted cell products. In the mid-1980s, approximately 5% of all marrow transplant recipients were reported to have adenovirus recovered [360, 366], and that number increased to 12–21% in the mid-to-late 1990s [367, 368].

In the BMT population, approximately 33% of infected patients develop severe and prolonged symptoms, such as (1) gastrointestinal infection, manifested as diarrhea, hemorrhagic colitis, or hepatitis; (2) urinary tract infections, manifested as hemorrhagic cystitis often associated with renal failure; and (3) pulmonary infections, resulting in interstitial pneumonia. Adenovirus infections in hematopoietic stem cell transplant (HSCT) recipients are often disseminated, which is defined as isolation of the virus from two different sites or from the blood [358, 365, 367, 369–373].

The BMT programs at both the University of Kentucky and the University of Wisconsin have reviewed the incidence of adenovirus infections in their transplant population [371, 373]. Using different definitions for infection and disease, these programs have established risk factors for adenovirus disease in BMT recipients. First, the age of the BMT recipient is predictive for infection, with pediatric patients more likely to have a positive culture for adenovirus than a similar adult population (23% vs. 9%, $p < 0.0001$). The reasons for this remain unknown, and the type of transplant procedure does not explain this finding. Second, the type of BMT predicts for infection. The Kentucky program demonstrated that allogeneic BMT recipients are more likely to develop infection as compared to autologous recipients (16% vs. 3%, $p < 0.0001$) [368]. Differences have been shown in some series among allogeneic patients, depending on the degree of HLA disparity. A higher incidence of both adenovirus infection and disease was seen in patients undergoing unrelated HLA-mismatched grafts compared with related HLA-matched grafts, although the difference was not statistically significant [363, 366]. Finally, adenovirus disease developed in a greater number of patients with moderate-to-severe GvHD (15 vs. 5 $p < 0.05$). The development of moderate-to-severe GvHD was found to be an independent risk factor predictive for adenovirus disease on multivariate analysis ($p < 0.01$; odds ratio = 5.4; 95% confidence interval 1.6–17.9). The other major risk factor for progression of infection to disease is isolation of adenovirus from multiple sites. Isolation of adenovirus from more than two sites strongly correlates with an increased risk of invasive disease compared to isolation from one site; adenovirus disease occurred in 63% of patients with more than two positive sites compared to 11% with only one positive site.

The development of invasive disease is associated with a shorter median survival. The median survival for all patients who had a positive adenovirus culture was 208 days. Patients with evidence of invasive infection had a median survival of 100 days, compared to 998 days for those with noninvasive adenovirus infection [373]. Of those patients who develop invasive infection, 50% die.

Treatment

Clearance of adenovirus has been shown to correlate with recovery of adenovirus-specific T-cell immunity [374, 375]. When possible, rapid tapering or withdrawal of immunosup-

pression is felt to be the best way to prevent progression of adenoviral infection [376–379]. Currently, there is no reliably effective antiviral therapy for the treatment of adenovirus infections. There are numerous case reports in the literature reporting success with ganciclovir [380–382], intravenous ribavirin [383–390], vidarabine [391, 392], and cidofovir [383–395]. The key to successful treatment is prompt initiation of therapy in high-risk individuals. A risk-adapted approach, similar to that taken with CMV, may in fact prevent progression to disseminated disease, which is typically associated with a dismal outcome. An alternative strategy that has been exploited is adoptive immunotherapy. There are data supporting the use of donor leukocytes to treat posttransplant EBV-associated lymphoproliferative disease, as discussed previously. This principle has been extrapolated to the treatment of life-threatening adenoviral infection following T-cell-depleted HSCT and has met with success, although there are only published data on one patient [396].

Clearly there is an absence of adequately controlled studies on treatment of adenovirus infections with the currently available antiviral agents. Cidofovir and ribavirin appear to offer most promise and should be further investigated in a prospective multicenter trial.

Prevention

In the allogeneic transplant population, patients can be stratified according to their risk of adenoviral disease:

Low. autologous HCT recipients [368, 397, 398]

Intermediate. T-cell replete, related-donor allograft recipients without GVHD

High. T-cell depleted (2–3 log) related or unrelated-donor transplants [383, 384]; HLA-mismatched transplants (other than DRB1 mismatch) [399]; patients with GVHD on systemic steroids [369, 370, 398], pediatric patients

Highest. refractory GVHD; unrelated cord blood or haploidentical stem cell graft, T-cell depleted (>2–3 log), use of anti-T-cell antibodies (antithymocyte antibody, alemtuzumab) [383, 400]

It is currently advised that patients in the highest risk category undergo weekly PCR monitoring for active adenovirus for either the first 6 months after transplant or for the duration of immunosuppression [399, 401]. Unfortunately, there is no definitive data on a critical value for viral load in the peripheral blood to indicate initiation of treatment.

Respiratory Viruses

Respiratory viral infections are a common cause of morbidity and mortality among patients with hematological malignancies. Despite significant advancements that have been made in our understanding of the pathophysiology, epidemiology, and laboratory diagnosis of these infections; improvement in available treatment regimens for established disease has been limited. A major focus has therefore remained on prevention. Along with adenovirus (already discussed previously) which can also present as an upper respiratory infection, respiratory syncytial virus, influenza, and parainfluenza are the most commonly encountered and will be discussed next [402–404].

Respiratory Syncytial Virus

Respiratory syncytial virus (RSV), a member of the paramyxoviruses, is a single-stranded RNA virus related to parainfluenza virus and is a well-recognized cause of upper and lower respiratory tract illness in children [405–408]. Outbreaks of RSV infection are seasonal and usually occur in the community during November to April, the fall and winter months in the Northern Hemisphere [409, 410]. Repeated infections are common in all age groups, and previous infection does not confer immunity and prevent subsequent infections, even in sequential years [411, 412].

The virus can be transmitted by three possible mechanisms [413]. The first is transmission by small-particle (less than 10 μm diameter) aerosols, which are typically produced by coughing or sneezing. These are able to traverse distances of 1.8 m or greater. Second is transmission by droplets or large particles. This type of transmission necessitates person-to-person contact, usually at distances of less than 0.9 m for infection to occur. Finally, transmission can occur via contact with contaminated surfaces. Typical winter conditions enable RSV to remain viable on nonporous surfaces for longer than 6–12 h. Therefore, assuming virus viability, there is great potential for self-inoculation and transmission of infection to surrounding patients and medical personnel. This is particularly worrisome for medical personnel and nursing staff, who may unwittingly spread the virus during high-risk months. Prevention using good infection control practices is of paramount importance. Nosocomial transmission has been implicated in some groups of immunocompromised patients [414]. Respiratory secretions are contagious and the portal of entry is through the nasopharyngeal mucosa and conjunctivae. The incubation period of RSV is 3–5 days. The target tissue is the respiratory epithelium. Proliferation and necrosis of bronchiolar epithelium with lymphocytic peribronchiolar infiltration and edema are characteristic pathologic findings. Both humoral and cell-mediated immunity are important protective responses [415], and those individuals with inherited or acquired immunodeficiency have more severe and long-lasting RSV infections than normal individuals [408].

RSV infections in BMT patients have been increasingly described since 1988, with several outbreaks being reported

in large BMT centers [399–408]. The reported incidence of RSV infections in hospitalized BMT patients ranges from 7 to 20%. High-risk groups for the development of RSV infections include premature infants; children with bronchopulmonary dysplasia, congenital heart disease, or cystic fibrosis; immunosuppressed patients, including bone marrow and solid organ transplant recipients and those with underlying disorders of cellular immunity; elderly people; and those living in institutions [409]. Children with hematologic malignancies are susceptible to RSV infection, which has the potential to cause more severe illness in these patients than in nonimmunocompromised patients [407, 416], and can result in extension to the lower respiratory tract, pneumonia, and death. Adults with hematologic malignancy are also susceptible [417, 418], especially those profoundly immunosuppressed after marrow transplantation.

In immunocompromised children and adults, RSV infections usually present as an upper respiratory tract illness with cough, fever, sinus and nasal congestion, rhinorrhea, and dyspnea but rapidly progress to severe and often fatal lower respiratory tract viral pneumonia, with marked radiographic changes [419]. A review of 600 children with RSV infection revealed 47 patients with compromised immune function over a 10-year time span. On analysis of the data, those children with primary immunodeficiency disorders and those receiving chemotherapy were more likely to develop RSV pneumonia (60–80% admitted to intensive care units) and had a higher mortality rate (15–40%) compared with those with normal immunity (5% intensive care unit admissions; fewer than 1% died) [407]. In the BMT population, lower respiratory tract pneumonia develops in approximately 50–60% of RSV-infected patients [410, 419]. The development of pneumonia is associated with a higher mortality rate (66–100%), and therefore rapid treatment and prevention of progression are important [409, 417, 420, 421]. The M.D. Anderson Cancer Center has produced data demonstrating that those patients treated earlier (1 day or less from symptoms) had an overall mortality rate of 22% versus 100% in patients who had delayed treatment or who did not receive treatment [417]. This confirms that the mortality rate increases as the time to diagnosis and treatment increases. The other known predictor of outcome in BMT recipients is engraftment status. The risk of pneumonia is greater in patients who have not engrafted than in those who have (79 vs. 41%) [419]. There are also data demonstrating that the frequency of progression from an upper respiratory tract infection to pneumonia is higher in patients in whom engraftment has not occurred or who are less than 1 month posttransplant, compared to those who are more than 1 month posttransplant/engraftment (70–80% vs. 25–40%, respectively). However, once pneumonia develops, engraftment status does not alter overall mortality [410, 420]. Other risk factors that have been identified as causing progression of RSV infections from an upper respiratory tract to a lower respiratory tract infection include older age and HLA-mismatched or unrelated donor transplants (relative risk 2.8.1) [422].

Diagnosis

Nasopharyngeal aspirates or washes are among the most common specimens used for the diagnosis of RSV. Wash specimens are preferred to swabs because they have increased sensitivity. Diagnosis can be made by molecular and nonmolecular methods. Specimens may be tested for RSV by culture or by use of a rapid detection assay such as enzyme-linked immunoabsorbent assay (ELISA) or immunofluorescence [408]. More sensitive methods such as PCR analysis for the presence of viral-specific nucleic acid have been more utilized with more recent attention to the quantitation of viral load. Specimens from sputum or bronchoalveolar lavage also contain the pathogen, and in the immunocompromised patient, bronchoalveolar lavage has an increased sensitivity for virus detection

Treatment

Currently there are a number of available treatment options for RSV pneumonia which include ribavirin, intravenous immunoglobulin rich in RSV titers, RSV immune globulin (RSV-IG [Respigam, MedImmune, Gaithersburg, MD]), and palivizumab (Synagis, [MedImmune, Gaithersburg, MD]). Unfortunately there are no large randomized controlled trials in patients with hematological malignancy supporting the efficacy of any one of these treatments alone or in combination. The most commonly employed agent, ribavirin is a synthetic purine nucleoside derivative [423]. Its antiviral effects result from three mechanisms of viral inhibition, namely, suppression of viral nucleic acid synthesis; blocking of the formation of the terminus of mRNA, leading to inefficient translation of mRNA into viral structural proteins; and suppression of viral polymerase, resulting in interference with viral mRNA expression and subsequent protein synthesis. Ribavirin has activity against a broad spectrum of both RNA and DNA viruses and has been investigated as a potential treatment in influenza A and B, disseminated adenovirus, parainfluenza virus, herpesvirus, and RSV infections. Ribavirin may be given orally (15–20 mg/kg/day divided into 2–3 doses) or aerosolized (6 g administered continuously or 2 g three times per day). Its role in RSV infections is controversial with much of the data on treating RSV infections come from the pediatric literature, as this is one of the populations most at risk for this infection. Conflicting results are reported on the use of ribavirin to treat RSV infections in infants and children. Several studies of infants and children on ventilators have examined the effect of ribavirin on days of ventilation and hospitalization. The results are variable, with one study showing a reduction in days of ventilation

and hospitalization [424], one study demonstrating no difference in hospitalization [425], and two studies showing prolonged hospitalization and ventilation compared to placebo [426, 427]. A meta-analysis of studies in infants with RSV lower respiratory tract infections demonstrated an overall trend in favor of ribavirin but no evidence of a significant benefit; unfortunately, the studies lacked the power to detect reductions in mortality [428]. Based on these data, ribavirin is not often used for infants and children with RSV infections and is reserved for immunocompromised and critically ill patients.

An abundance of case reports and small series report the effectiveness of aerosolized ribavirin in the treatment of RSV in both adult and pediatric BMT recipients [409, 417, 429–432]. It is important to remember that the number of patients treated is small and the studies are uncontrolled and noncomparative. One of the largest studies of RSV infection in BMT recipients reported 31 cases of RSV infection diagnosed among 199 transplant recipients [409]. Of the 31 patients, 18 were diagnosed with RSV pneumonia and the remaining 13 had upper respiratory tract infections. Ribavirin was administered to 13 (72%) of the patients with pneumonia but to none of the patients with upper respiratory tract infections. Survival among patients who developed pneumonia was poor, with a mortality rate of 78%. The mortality rate was 70% in the 13 patients treated with aerosolized ribavirin monotherapy and 100% in the untreated patients. Responses appeared to be better in those patients receiving more than 5 days of ribavirin therapy. An earlier study also documented a mortality rate of 66% in BMT patients with radiographically documented RSV pneumonia treated with aerosolized ribavirin monotherapy [421]. Based on these findings and the results outlined in case reports in the literature, it is difficult to justify monotherapy with ribavirin for the treatment of RSV pneumonia in immunocompromised individuals. If ribavirin is to be effective, early diagnosis is essential and treatment should be instituted prior to progression to pneumonia. In a randomized controlled trial, the efficacy and tolerability of aerosolized ribavirin for preventing the progression of RSV upper respiratory tract infection (URI) to a lower respiratory tract infection (LRTI) was studied in 14 patients who had undergone myeloablative conditioning and were 90 days post-HSCT [433]. Patients were considered to be infected if they had upper respiratory signs, respiratory rate greater than 150% of baseline, increasing cough, wheezing, sputum production, or pleuritic chest pain and oxygen saturation less than 90% on 2 consecutive measurements and a documented RSV infection. Patients randomized to drug therapy received aerosolized ribavirin 2 g (over 2 h) three times each day for 10 days. The primary outcome was progression to clinical pneumonia. Following randomization, 9 patients were allocated to treatment and 5 patients received supportive care. Clinical pneumonia after randomization occurred in 1/9 (11.1%) ribavirin patients and 2/5 (40%) of control patients ($p = 0.51$). There were no deaths in the trial. Overall, this study did not reveal any differences in outcomes despite trends in lower viral loads among ribavirin recipients.

In a study by Chemaly et al., the effect of aerosolized ribavirin on preventing pneumonia in 107 HSCT or hematologic malignancy patients with RSV upper respiratory infection was reported [434]. Patients received 6 grams of aerosolized ribavirin through a face mask inside a scavenging tent for 18 h/day (duration of therapy not defined). In this study, the authors identified 107 RSV patients, 61 were treated with ribavirin and 46 were provided supportive care. Progression to pneumonia occurred in 12/61 (20%) and 27/46 (59%) of patients, respectively ($p < 0.005$). On multivariate analysis, patients who developed pneumonia were older (OR, 1.037; 95% CI, 1.001–1.074; $p = 0.042$) and did not receive RSV-directed antiviral therapy (OR, 4.67; 95% CI, 1.20–18.10; $p = 0.025$). In addition, degree of lymphopenia (absolute lymphocyte count <200 cells/mL) was associated with poor outcomes and should be included in the treatment algorithm.

A number of small studies have suggested improved response rates and improved survival rates with combination therapy [435]. Whimbey et al. [436] studied aerosolized ribavirin 20 mg/mL for 18 h/day in combination with IVIG 500 mg/kg every other day in BMT patients who were RSV positive. The IVIG product was chosen based on the presence of neutralizing antibodies against RSV subtypes A and B. Of 42 patients who presented with upper respiratory tract illness, 19 were RSV positive. Pneumonia developed in 16 patients, of whom 12 (75%) were treated with combination therapy. The overall mortality rate among those with pneumonia was 42%, a significant improvement over mortality rates associated with ribavirin monotherapy. As seen with ribavirin monotherapy, responses were more likely to occur with fast implementation of treatment. The same group from Texas used the same combination of drugs (ribavirin plus IVIG) in a trial evaluating efficacy in preventing progression of RSV upper respiratory tract infection to pneumonia [420]. Fourteen patients were treated for a mean of 13 days with combination therapy. The upper respiratory illness resolved in 10 patients (71%). Four patients (29%) developed pneumonia, which was fatal in two. In this study, two different methods of administering ribavirin were evaluated. Traditionally, aerosolized ribavirin is administered at a daily dose of 6 g, delivered at a concentration of 20 mg/mL for 18 h/day in a scavenging tent to prevent environmental contamination with the drug [420]. Although it produces very high drug levels in bronchial secretions with little systemic absorption [437, 438], this method of administration is cumbersome and is often difficult for patients to tolerate for long periods. In an attempt to improve compliance and ease of

administration, short-duration aerosolized ribavirin at a daily dose of 6 g, administered at a concentration of 60 mg/mL for 2 h every 8 h was evaluated. This method of administration has been attempted in the past with limited success [419], but the favorable results reported by the Texas group offer promise and should be further evaluated in a larger group of patients.

The doses of standard IVIG necessary to achieve the required in vivo RSV-neutralizing activity are large, and frequent dosing is required. This, coupled with wide variations in RSV antibodies between batches and commercial sources, led to the development of a commercial RSV hyperimmune globulin. RSV-IG, a polyclonal hyperimmune globulin containing high titers of RSV antibodies (over 19,200 MU/mL) against RSV [439]. The product is commercially available as Respigam (MedImmune, Gaithersburg, MD).

The only available data in immunocompromised patients are in the BMT literature. The results of a compassionate use protocol of RSV-IG in the treatment of lower respiratory tract RSV infections in pediatric BMT patients showed that 55% of patients had resolution of lower respiratory tract infections following one dose [439]. Importantly it must be noted that of the patients enrolled in the program, 82% had previously received aerosolized ribavirin and 91% were receiving concurrent aerosolized ribavirin with RSV-IG. Despite concomitant treatment, the mortality rate was only 9%, and this offers promise for the future. Clearly, further studies using RSV-IG as monotherapy and comparing outcomes to those obtained with ribavirin are warranted in the immunocompromised patient population.

In addition to RSV-IG, a humanized RSV monoclonal IgG1 monoclonal antibody that is highly active against RSV types A and B is commercially available [440]. Palivizumab is approved by the US Food and Drug Administration for the prevention of serious RSV lower respiratory tract disease in pediatric patients at high risk for RSV disease. In the pediatric population it has been shown to reduce hospitalizations as a result of RSV infection by 55%, reduce the total number of days in hospital as a result of RSV infection (36 vs. 63 days), reduce the number of ICU admissions (1.3% vs. 3%), and reduce the total number of days hospitalized overall (191 vs. 242 days) [441]. In view of these promising data and the increasing incidence of RSV infection among immunocompromised patients, the use of palivizumab has been evaluated in a small number of BMT patients. There are two small studies in this patient group, one examining the pharmacokinetic profile of palivizumab in patients without RSV infection, and the other in patients with documented RSV infection. In the pharmacokinetic study, the mean serum concentration after 30 days was 41.9 μg/mL [442], which was within the limits necessary to produce a two-log (99%) reduction in pulmonary RSV infection, based on animal data [443]. Patients were also monitored for the development of antipalivizumab antibodies. No antibodies were detected, which is consistent with the pediatric literature [442]. The second study evaluated the response to palivizumab in 15 patients with proven RSV infections. At enrollment, 12 patients (80%) had lower respiratory tract involvement and 3 (20%) received mechanical ventilation. Palivizumab 15 mg/kg was administered in combination with ribavirin therapy. The survival rate was 87%, similar to results seen with RSV-IG. Palivizumab is an interesting addition to the therapeutic armamentarium against RSV and offers the advantage of being administered once and producing high concentrations that are sustainable and protective over a 30-day dosing interval. To date there are no comparative studies of RSVIG and palivizumab, so which one is superior remains to be seen.

McCoy et al. recently reported their single-center experience with a RSV algorithm developed by interdisciplinary team for managing RSV infections in 26 patients with hematologic malignancies [444]. Practice guidelines were used to stratify patients by disease severity, time post-HSCT, and degree of lymphopenia. Patients with URI and less than 1 month post-HSCT and/or absolute lymphocyte count <300 cells/mm^3 received aerosolized ribavirin (6 g over 12 h for at least 3 days). If patients presented with LRTI, ribavirin and palivizumab (15 mg/kg for one (1) dose) were administered. Upon review of the data, 13 patients presented with URI and 13 patients presented with LRTI. The average number of ribavirin doses was 5 ± 2.1. No mortality occurred within 30 days of RSV diagnosis. No other variables were addressed regarding the efficacy of the regimen.

Khanna et al. also published the experience from their center along with a review of the literature focusing on the treatment of RSV infections in patients with hematological diseases [445]. The authors of this study identified 780 RSV-infected patients from 1981 through 2007. Aerosolized ribavirin was administered to 337 patients with URI and LRTI. In patients with URI, 30/95 (32%) and 76/112 (68%) progressed to LRTI for ribavirin treated and untreated patients, respectively. In patients with LRTI, death occurred for 39/102 (38%) of patients treated with aerosolized ribavirin, 50/116 (43%) treated with combination ribavirin and IVIG, 15/36 (42%) of untreated patients. These data suggest that early treatment may play a role in reducing progression to LRTI and that treatment may have minimal benefit once disease has progressed beyond URI. RSV infections are not only amenable to aerosolized therapy but may be treated with oral therapy. Oral ribavirin is rapidly absorbed and has been evaluated in several reports. Khanna et al. performed a retrospective analysis on the efficacy of oral ribavirin ± immunoglobulin (IVIG) and/or palivizumab antiviral agents in 34 patients with RSV infection and hematological diseases. Patients were stratified based on degree of immunosuppression and the presence of URI or LRTI. Patients were strati-

fied into moderate or severe immunosuppression based on time from transplant, time from T- or B-cell depletion, presence of lymphopenia, or GvHD grade ≥ 2. Patients with moderate immunosuppression received oral ribavirin (1200–1800 mg/day) plus IVIG (once a week). Patients with severe immunosuppression with URI or LRTI received oral ribavirin plus IVIG plus palivizumab. At the time of RSV diagnosis, 22/34 had URI and 12/34 had LRTI. In patients with URI, 10/22 had moderate immunosuppression and none of the 10 progressed to LRTI. In 12 patients with severe immunosuppression and URI, 2/12 progressed to LRTI. In patients with LRTI and severe immunosuppression, five (5) out of 10 died. For those patients who died, degree of immunosuppression and the presence of LRTI were associated with a poor outcome. RSV loads in nasal secretions were evaluated in 19 patients. A greater than 2 log10 copies/ml drop from baseline was seen in 11/19 within 7 days. None of these patients went on to develop LRTI. Overall, oral antiviral therapy was most effective in preventing disease progression in those patients with moderate immunosuppression and URI.

Avetisyan et al. evaluated the role of systemic ribavirin (intravenous or oral at 15–20 mg/kg/day) therapy in 28 HSCT recipients with laboratory verified RSV infection [446]. No adjunctive therapy was administered (e.g., palivizumab). Following treatment, all patients with URI did not progress to LRTI and survived. For patients with LRTI, 5/14 died directly from RSV. Overall mortality was approximately 20% most of which was in those patients with LRTI. Of note, these data are similar to other reports in which broader antiviral therapy was applied. Overall, these data speak to the fact that degree of immunosuppression and presence of LRTI are associated with poor outcomes and may not be amenable to combination therapy.

With mortality rates approaching 40–60% in patients with lower respiratory infection, ribavirin has become an option for treating patients with upper respiratory disease in the hope of minimizing disease progression. Currently available data suggest that early recognition of infection with RSV, supportive care measures, such as oxygenation and hydration, and reduction of immunosuppressive therapy if possible may be beneficial in improving outcome in improving outcomes. Antiviral therapy appears to be most effective when it is started within 48–72 h of symptom onset, although there are no large randomized controlled trials available to help determine the optimal treatment regimen. With low-level evidence, most data suggest that ribavirin is probably beneficial in preventing disease progression in patients with symptoms of upper respiratory infection. Unfortunately, the initiation of antiviral therapy once lower respiratory infection has developed has not shown a significant benefit in reducing the risk of RSV-related mortality with or without an additional immunotherapy in the form of IVIG or monoclonal antibody.

Although it appears that patients undergoing therapy for hematological malignancy with upper respiratory infection with RSH may benefit from treatment with ribavirin by preventing disease progression, a number of questions regarding treatment remain. One such question is the appropriate route of administration of the drug, particularly in light of the challenges in delivery of the aerosolized formulation of ribavirin. In the US, aerosolized ribavirin is the only FDA-approved agent available for the treatment of RSV approved in 1985. Therefore, most early studies in patients with hematologic malignancies utilized this form of the drug. Since then, aerosolized administration optimized drug concentrations at the site of infection interest stayed with the aerosolized product. There are reasons, however, to consider that oral ribavirin could replace aerosolized therapy in managing respiratory viruses in patients with hematological malignancy. Ribavirin is rapidly absorbed with a reasonable bioavailability at 64% (extensive first pass metabolism), has a large volume of distribution due to tissue and intracellular penetration, and has data to support its role in reducing disease progression (similar to inhaled). In addition, oral therapy is safer for healthcare workers (avoids environmental exposure) and significantly less expensive (Avetisyan). Well-controlled clinical trials to better define the appropriate antiviral management of infection with RSV in this patient population are warranted.

Prevention

Patients presenting for BMT during the RSV season with respiratory symptoms should receive workups for conventional respiratory viruses (CRVs), including RSV, adenovirus, and influenza and parainfluenza viruses. If any of these viruses are isolated, the BMT should be placed on hold until resolution of the acute process if possible. Similarly, patients with cancer undergoing chemotherapy should have therapy temporarily suspended if possible [134].

If any hospitalized BMT patient develops signs and symptoms of CRV infection while an inpatient, appropriate specimens should be obtained for viral culture and rapid diagnostic tests for RSV [437]. If the patient continues to display respiratory signs and rapid testing is negative (two diagnostic samples more than 2 days apart), a bronchoalveolar lavage should be considered. These patients should be placed under contact precautions to prevent spread of infection.

Healthcare workers (HCWs) handling respiratory symptoms of infected patients should wear gowns, surgical masks, and eye protection to avoid contamination from secretions. Protective clothing should be put on when entering the patient's room and discarded before leaving the room. HCWs should change gloves between contacts with patients. All visitors of an infected patient should wash their hands after contacts with the patient or with secretions from the patient. In addition, visitors with upper respiratory tract infections

should defer visiting until resolution of symptoms, and HCWs with respiratory symptoms should be reassigned to duties other than patient care. Unfortunately clinical trials with inactivated and attenuated vaccines have not proven benefit. Further study of subunit vaccines are under development.

Influenza Viruses

Influenza A and B viruses are members of the Orthomyxoviridae family. These enveloped single-stranded RNA viruses are important causes of recurrent epidemics of respiratory disease in both normal and immunocompromised patients. Four genera exist. Influenzavirus A, influenzavirus B, influenzavirus C, and thogotovirus [438]. Influenza A viruses are further divided into subtypes on the basis of serological and genetic differences in their surface glucoproteins [438]. Fifteen subtypes of hemagglutinin (H1 to H15) and nine subtypes of neuraminidase (N1 to N9) have been identified. Considerable antigenic variations can occur; this property has made control by vaccine difficult because these antigenic changes can permit the organism to evade established immunity. Influenza viruses are named by type, location of isolation, number of isolates, year of recovery, and for influenza A, the subtype (e.g., A/Texas/36/91[H1N1]) [447]. Three influenza A virus subtypes, H1N1, H2N2, and H3N2, have caused extensive human diseases during the last century.

Infected respiratory secretions are generally the mode of transmission to a susceptible individual. The target cells are those of the respiratory tract epithelium, particularly the columnar epithelial cells, where the influenza virus replicates [447]. Desquamation of epithelial cells, sloughing and in more severe cases, hemorrhage, hyaline membrane formation, and neutrophilic infiltration are notable findings pathologically. Influenza typically presents clinically as fever with associated chills. Other common presenting symptoms include sore throat, headache, myalgias, and fatigue. In more severe cases patients may present with fulminant pneumonia, a common presentation in the elderly in influenza season. The incubation period for influenza viruses is between 1 and 4 days. Secondary bacterial infections (mainly *Streptococcus pneumoniae*, *Staphylococcus aureus*, and *Haemophilus influenzae*) are well-recognized complications. Humoral immunity is an important protective host response, especially neutralizing antibody.

Influenza infections in immunocompromised patients occur, concomitant with outbreaks in the community. For example, an influenza A epidemic in 1991–1992 in Houston was evaluated [448]. Among adult BMT patients, approximately 40% developed acute respiratory illness, and influenza A was isolated from 30%. All had upper respiratory tract illness, but this was complicated by pneumonia in 75%. The case fatality was 17%. Nosocomial acquisition of influenza is also common in hospitalized adults with leukemia or those undergoing BMT [448–450]. Influenza infections in immunocompromised patients have been studied regularly over the past decade and a review of CRVs recovered from 785 consecutive immunocompromised patients with suspected respiratory tract infections has been published [451]. Diagnosis of CRVs was made following bronchoalveolar lavage in all cases, and 199 viruses were isolated from these specimens (23%). Influenza virus was isolated in 11 of 37 patients with CRVs and was the most common CRV isolated. Pneumonia was present in 82% of these patients, and the overall mortality rate was 27%. These results are similar to those previously published in the literature [418, 452] and indicate the need for stringent preventive measures during influenza season and community outbreaks.

In April 2009, an outbreak of a novel H1N1 influenza A virus was noted in Mexico which had a reportedly high mortality rate in an otherwise “low-risk” patient population [453]. This virus was noted to be a reassortment of DNA including genetic components of Swine and human influenza [454]. As opposed to the seasonal influenza virus, mortality from the newly designated Influenza A/pandemic 2009/H1N1 appeared to be higher in infants, young adults, those with obesity, and pregnant women. It was postulated that older individuals may have had some cross reactive immunity to prior exposure of to strains of influenza A not shared by younger individuals [455]. Shortly after infection with this virus was noted in the United States [456], and two cases of infection with the novel H1N1 (“Swine flu”) virus in allogeneic hematopoietic stem cell transplantation recipients were reported [457]. In those cases, prolonged viral shedding with eventual development of resistance to oseltamivir was noted. One patient improved without change in antiviral therapy and one was treated with zanamivir and ribavirin with persistent illness and prolonged hospitalization. Additional cases have been reported in patients with hematological malignancy and/or undergoing hematopoietic stem cell transplantation with varying outcomes [458]. Although pandemic in its spread across the globe, significant increase in influenza-related mortality like that seen in the 1918 outbreak was not noted; however, this outbreak was a warning to healthcare officials and the general public as to how rapidly a contagion can spread globally.

Detection

There are four broad potential targets in diagnosing influenza [459]: virus isolation, detection of viral proteins,detection of viral nucleic acid, and serological diagnosis. The gold standard diagnostic procedure for influenza is isolation in cell culture. This procedure requires 2–4 days to produce a result. The shell vial technique is much faster, enabling detection of influenza antigens following overnight culture, although the

sensitivity of this test is only approximately 80% as compared to standard cell culture [459]. A new area of emerging diagnostics is the detection of viral proteins. There are several rapid tests now on the market, which are easy to perform and do not require skilled technicians. Point-of-care rapid diagnostic tests that are currently available or under development include the Directigen Flu A test, which detects only influenza A in nasopharyngeal, nasal, or throat specimens. This is an enzyme immunoassay test and can be completed in eight steps in approximately 15 min. The sensitivity of the test is variable, ranging from 50 to 99%, with a specificity of 52–97% [438, 447]. The other tests are the ZstatFlu, FLU OIA, and QuickVue. Each of the three latter tests can detect both influenza A and B. The ZstatFlu is a colorimetric neuraminidase enzyme assay that has only three steps but takes 12 min to produce a result from a throat specimen. It has a sensitivity of 62% and a specificity of 99%. The FLU OIA assay method is an optical enzyme immunoassay, which can be completed in seven steps. It is rapid, with a 17-min turnaround, a sensitivity of 62–88%, and a specificity of 70–80%. The QuickVue takes the longest of all the new techniques; it is able to produce a result from a nasopharyngeal or nasal specimen in 30 min and has a sensitivity of 73–82% and a specificity of 96–99%. These tests are rapid and provide quick answers to assist therapeutic decisions, although they are not as sensitive as culture or PCR techniques. In addition, these tests have not been evaluated in the immunocompromised population and the sensitivity and specificity parameters listed are based on studies performed in healthy children and adults. Although these rapid diagnostic tests assist in early treatment, they will not replace the need for viral culture because only culture isolates can provide specific information on circulating influenza subtypes and strains [460]. This information is necessary annually to compare circulating influenza strains and vaccine strains and to formulate vaccines for the coming year.

PCR techniques detect viral nucleic acid in a very sensitive manner, and results can be generated within 24 h. This diagnostic tool is best used in outbreak situations, in which molecular typing and analysis of samples is necessary. Finally, serological diagnosis is based on the detection of a fourfold or greater increase in antibody titers. The test requires two samples, one collected at the onset of symptoms and another 14 days later, and this limits its usefulness in guiding treatment [418]. This test is not as reliable in the immunocompromised population.

Treatment

Amantadine and rimantadine are agents that inhibit an early stage of viral replication and appear to be useful in the prevention and treatment of influenza A in young adults, children, families, and the elderly at risk for exposure [461–465]. Both these drugs act to inhibit the uncoating of the influenza A virus (no activity against influenza B virus) by blocking the activity of the viral M2 protein [460]. Both agents are similarly efficacious (70–90%), although rimantadine may be associated with fewer side effects [466]. These compounds exert maximal benefit when administered within 48 h of the onset of influenza symptoms. They have been shown to reduce the duration and severity of symptoms when administered in this way, although they have not been shown to prevent serious influenza-related complications such as bacterial or viral pneumonia or exacerbation of chronic diseases. One of the main concerns with the use of amantadine and rimantadine is the emergence of drug resistance. Resistance to these agents has been well documented in the laboratory and in humans [467–470], but the frequency and importance of drug-resistant influenza in immunocompromised patients was uncharacterized until recently. It has since been shown to occur in approximately 30% of severely immunocompromised patients with influenza and in 83% of patients with symptomatic disease who shed virus for more than 3 days [467]. In patients who do not respond to treatment, resistant strains must be suspected. Toxicity is also a concern with this class of agents.

A second class of antiviral drugs, the neuraminidase inhibitors, is also available for the treatment of influenza A and B [471–475]. Zanamivir and oseltamivir are both commercially available and FDA approved for the treatment of influenza A and B. They are oral agents and are better tolerated than amantadine and rimantadine. They are not approved for, nor are there currently sufficient data to support their use in the prophylactic setting. Despite this lack of data, in time of community or nosocomial outbreaks of influenza B infections it may be prudent to prophylactically administer these agents to high-risk individuals; this approach certainly deserves further study. These drugs act by selective inhibition of viral neuraminidase. Neuraminidase, essential for viral replication in vitro, promotes the release of progeny virus from infected cells [461]. By inhibiting neuraminidase, zanamivir and oseltamivir block the active site of neuraminidase and leave uncleaved sialic acid residues on the surface of host cells and influenza viral envelopes [461]. Viral hemagglutinin binds to the uncleaved sialic acid residues, resulting in viral aggregation and a reduction in the amount of virus released [460].

Zanamivir is available for use by inhalation only, using the Diskhaler provided in the package. It is approved for the treatment of influenza in patients 12 years and older who have had symptoms for no longer than 2 days. The recommended dose is two inhalations (10-mg dose) twice daily for 5 days. Following inhalation, approximately 7–21% of the administered dose reaches the lungs, with 70–87% of the dose being deposited in the oropharynx [460, 472]. A small amount of the drug is absorbed systemically (4–17%). Zanamivir has an advantage in that the dose does not need to

be modified in renal impairment. Oseltamivir is available as an oral capsule. It is approved for the treatment of influenza A and B virus in adults 18 years of age or older within 2 days of the onset of influenza symptoms. The recommended dose for adults is 75 mg by mouth twice daily for 5 days. Doses need to be reduced in patients with creatinine clearances less than 30 ml/min to 75 mg daily Approximately 80% of a dose is systemically absorbed [460]. Both of the commercially available neuraminidase inhibitors have favorable side effect profiles. The most common side effects seen include nausea, sinusitis, nasal signs and symptoms, diarrhea, cough, dizziness, and headaches. The majority of these side effects could be attributed to the underlying influenza infection and were seen with a similar incidence in the placebo groups of the earlier trials. Both neuraminidase inhibitors have been shown in clinical trials in healthy volunteers to reduce the symptoms associated with influenza by approximately 1 day. There is currently a lack of data supporting the use of these agents in immunocompromised patients, patients who are hospitalized, or patients with severe influenza including pneumonia.

As seen with amantadine and rimantadine, resistance to neuraminidase inhibitors can be induced. The emergence of resistance, however, is much less likely to happen, requiring several passages in cell culture [460], whereas resistance to amantadine and rimantadine requires far fewer passages in cell culture. Resistance to zanamivir has already been documented in a BMT recipient [476], and therefore the use of these agents in this population warrants close surveillance. In addition, osteltamivir-resistant novel influenza H1N1 virus infection has also been reported during the recent outbreak [457].

Machod et al. described the benefit of early administration of antiviral therapy in HSCT recipients with the administration of oseltamivir within 48 h of initial symptoms likely to prevent more serious disease [477]. The optimal duration of therapy for patients who develop influenza infections, however, remains unanswered. Prolonged viral shedding leads to the potential of development of drug resistance. In a report by Khanna et al., 18 of 21 episodes of influenza in allogeneic transplant recipients were treated with 75 mg of oseltamivir twice daily for a median of 11 days (8–14) and all survived [478]. Recurrent viral shedding and need for retreatment has also been described [479].

Prevention

Previously, amantadine and rimantadine were used primarily as chemoprophylaxis for community and nosocomial outbreaks of influenza A. Their use has largely been supplanted by the neuraminidase inhibitors. It is currently advised that during community or nosocomial outbreaks, the following stem cell transplant populations should receive chemoprophylaxis with a neuraminidase inhibitor [134]:

1. Transplant recipients less than 6 months after transplant.
2. Transplant recipients who have not received a current influenza vaccination and are greater than 4 months after transplantation.
3. Transplant recipients who are less than 24 months after transplant or who are greater than 24 months after transplant and substantially immunocompromised who have been exposed to influenza. Additionally caregivers and close contacts of transplant recipients should also receive chemoprophylaxis if an outbreak occurs with a strain of influenza not contained in the available influenza vaccine [134].

Inactivated vaccines are available, and community immunization programs in the fall and early winter can reduce the spread within communities. The efficacy of the influenza vaccine in cancer patients and the severely immunocompromised host is reduced as compared to normal controls. Several attempts to overcome poor immune responses, including administering additional injections, have been made although they have not met with much success. Patients with mild underlying immunodeficiency have been able to mount an antibody response. The scenario in BMT patients is somewhat different. In an evaluation of antibody responses to a two-dose regimen of influenza vaccine in allogeneic T-cell-depleted and autologous BMT patients, the likelihood of response was significantly related to a longer interval between transplantation and immunization [480]. Vaccination during the first 6 months unfortunately was ineffective. Similarly, patients with GvHD were less likely to undergo seroconversion. Thus, early after transplant an agent such as a neuraminidase inhibitor may be more useful in protecting susceptible patients than immunization. It is currently advised that all stem cell transplant candidates and recipients receive a seasonal trivalent inactivated vaccine [134]. In addition, vaccination of family members and close or household contacts is recommended during each influenza season. This immunization schedule should start the year prior to the BMT and continue for at least 24 months after BMT, generally for as long as the patient is immunosuppressed [134]. If the immunization occurred during an influenza outbreak, chemoprophylaxis, if feasible, should be provided for 2 weeks after influenza vaccination while the immunologic response to the vaccine develops.

Recommendations for vaccination and chemoprophylaxis are identical for autologous and allogeneic transplant recipients.

Parainfluenza Viruses

Human parainfluenza viruses (PIVs) are enveloped, single-stranded RNA viruses, which are members of the Paramyxovirus family (along with RSV, measles, and

mumps). Of the four major antigenic serotypes, three are common respiratory pathogens frequently causing upper (type 1 and 2) and lower (type 3) respiratory illness in the general population. Infections are seasonal, especially during the fall. The target tissues are the mucosal epithelium of the nasal and oropharyngeal passages, but infection can extend to bronchi and bronchioles in more severe cases. Neutralizing antibody and cytotoxic T-cell responses are immunoprotective. The virus can be isolated from infected secretions by cell culture techniques coupled with immunofluorescence.

Incidence and Outcomes

Parainfluenza virus is a frequent cause of respiratory illness in children less than 6 years of age. The disorder presents with a variety of symptoms, ranging from upper respiratory tract infections to croup and pneumonia [481]. By 30 months of age, nearly every child will encounter this virus, but immunity is incomplete and reinfection occurs throughout life. When reinfection occurs in later life, the infection course is generally less severe. Parainfluenza virus infections have been reported following BMT [481–484]. The reported incidence among BMT and cancer patients has ranged from 2 to 5% of patients. The largest review of PIV in BMT patients was conducted over 16 years from 1974 to 1990 at the University of Minnesota. Over 1200 patients were screened for common respiratory viruses, and PIV was isolated in 27 patients by either nasopharyngeal cultures or bronchoalveolar lavage. Approximately 30% of patients had respiratory symptoms localized to the upper respiratory tract, characterized by fever, cough, coryza, wheezing, and shortness of breath. The remaining 70% developed lower respiratory tract infections, of which 32% developed respiratory failure. Diffuse pulmonary infiltrates on chest X-ray and sinus opacification, in addition to cough, fever, wheezing, and shortness of breath characterized lower respiratory tract infections. As with other CRVs, development of pneumonia is associated with a poor outcome, with the majority of patients dying. This is in contrast to infection localized to the upper respiratory tract, which all patients survived. Unlike other CRVs such as RSV, infection with PIV was not seasonal but occurred in nearly every month of the year. The most common subtype identified was parainfluenza type 3, which is consistent with other reports in the literature.

The M.D. Anderson group has also reviewed the frequency and outcomes of PIV infections in their cancer and BMT population. Parainfluenza was isolated in 3% of 265 adult BMT patients during 1991. Again, PIV type 3 was identified in all patients. Progression to lower respiratory tract infections was associated with a 50% mortality. More recently, this group published updated results spanning a 5-year period. Parainfluenza was isolated in 61 patients (5.2% of the total screened population). Almost half of the patients evaluated with PIV developed pneumonia, with an associated mortality rate of 34%.

Based on these studies, it is apparent that patients at greatest risk for PIV infection are those who have recently undergone BMT, that is, who are less than 100 days from BMT, and those undergoing allogeneic BMT. In addition, patients with pneumonia were more likely to be lymphopenic than patients with localized upper respiratory tract infections (78% vs. 55%, $p < 0.005$). Other risk factors evaluated but not found to be statistically predictive for PIV pneumonia were patient age, presence of GvHD, type of immunosuppression, and the presence of neutropenia [484].

Treatment

There is no known effective antiviral treatment, although ribavirin may have some activity [401–403, 433, 485]. Ribavirin has been used with mixed results in PIV infections. The Minneapolis group treated nine patients (33% of the total PIV-infected population) with aerosolized ribavirin for a median of 10 days. The survival among those patients (78%) was no different from that patients not treated with ribavirin. This study was uncontrolled and was not intended to test the value of ribavirin therapy. The Texas group also treated some patients with ribavirin, achieving success in 60% of patients. At this juncture the role of ribavirin in PIV infections is unclear. Its role should preferably be in those patients with upper respiratory tract infections to attempt to prevent progression to a lower respiratory tract infection. However, in patients who have already developed a lower respiratory tract infection and are critically ill, it is also worth attempting. There are also limited data supporting intravenous ribavirin in the management of PIV infection [486].

Metapneumovirus

Human metapneumovirus is a single-stranded RNA paramyxovirus that causes respiratory tract infections in children, the elderly, and immunocompromised adults [487–490]. It has been identified as the cause of upper and lower respiratory tract infections worldwide during the late winter months (January to April). In one study of lung transplant recipients, infection was found in bronchoalveolar lavage fluid of 5% of symptomatic patients [487].

Infection manifesting as acute pneumonia with diffuse alveolar damage and hyaline membrane formation has been described in lung and heart lung transplant recipients [491, 492]. In BMT recipients, one study isolated human metapneumovirus by RT-PCR in 26% of symptomatic patients undergoing bronchoscopy and was associated with an 80% mortality rate [493]. Infection usually occurred within the first few weeks after transplant and was characterized by fever, nasal congestion, cough and the rapid development of

hypoxia, hypotension, and a rapidly worsening pneumonia. Pleural effusion and nodular infiltrates can be seen, which can help differentiate the infection from RSV. Prolonged asymptomatic infection after BMT has also been described [494]. Ribavirin has been demonstrated to decrease human metapneumovirus replication in the lungs in a mouse model [495]. Intravenous ribavirin has been effective in the treatment of metapneumovirus infection in lung transplant recipients [496].

Hepatitis Viruses

Hepatitis viruses are RNA (hepatitis A, C, and G) or DNA (hepatitis B) enteric pathogens, which can be transmitted via the fecal–oral route or sexual contact. Transmission through infected blood products is also problematic and represents a serious challenge for the management of patients with hematologic malignancies requiring blood product support. A summary of recommendations for the management of the various hepatitis viruses are listed in Table 51.6.

Hepatitis A

Hepatitis A is a member of the Picornaviridae family. This nonenveloped single-stranded RNA virus replicates in the intestinal tract and then is transported to the liver, which is the major target tissue. It can cause acute hepatitis characterized by jaundice or can present subclinically. In contrast to hepatitis B and C, only rare cases of hepatitis A transmission by blood products have been described, and therefore routine screening of blood products for hepatitis A virus does not occur. Transmission of the virus is thought to occur via the oral–fecal route. In addition, hepatitis A has a long incubation period of 15–50 days, with the virus excreted in the feces for 1–2 weeks prior to the onset of clinical illness and for at least 1 week afterwards [497], which emphasizes the need for good hygiene practices. The typical presentation of hepatitis A virus infections includes fever, malaise, nausea, vomiting, arthralgias, and anorexia [498]. In some patients flu-like symptoms may also be present. These symptoms tend to abate with the onset of jaundice. Rarely, acute liver failure, cholestatic hepatitis, or relapsing hepatitis can occur.

Table 51.6 Recommendations for screening, surveillance, and treatment of Hepatitis viruses

Virus	Screening	Surveillance	Prophylaxis	Treatment
A	Recipient	N	N	IVIG, Hep A vaccine
B	Donor/ recipient	Y	Y	Lamuvidine, famciclovir
C	Donor/ recipient	Y	N	Direct acting antivirals
G	N	N	N	Unknown

Management of acute hepatitis A infection is primarily symptom control. In otherwise healthy patients, resolution of the infection will occur in 3–6 months. The primary control measure is prevention. Patients traveling to hepatitis A endemic areas should receive either IVIG prophylaxis or hepatitis A vaccination. Similarly, immunocompromised patients who come into contact with hepatitis A-infected patients should consider prophylaxis. Passive immunization with IVIG has been the primary treatment of choice for over 50 years. Intravenous immunoglobulin is most effective when given within 2 weeks of exposure and is the fastest way to achieve hepatitis A antibodies. The alternative to IVIG is hepatitis A vaccine, of which there are two types used around the world, including the live attenuated vaccines and the inactivated vaccine. In the United States there are two commercially available inactivated vaccines, Havrix (Glaxo Smith Kline) and Vaqta (Merck). These vaccines produce very high levels of antihepatitis A virus, significantly higher than can be achieved with IVIG [498]. Following the first dose, antihepatitis A virus is detectable in the serum within 15 days in most patients. In the majority of patients, seroconversion is complete 1 month following injection, and persisting antibodies can be detected at 1 year. As with other vaccinations, immunosuppressed patients tend to be less responsive.

Indications for immunization include travel to endemic areas, chronic liver disease, and clotting factor disorders likely to require concentrate replacement. At this time, routine hepatitis A immunization is not recommended for BMT patients [446]. There are limited data on the safety of the vaccine in this population; however, consideration can be given to administration of the hepatitis A vaccine to patients older than 2 years who are at least 12 months post-BMT and who are at increased risk of for hepatitis A and its adverse consequences (e.g., persons with chronic liver disease, including chronic GvHD, and children living in areas with consistently elevated hepatitis A incidence) [497].

Testing for Hep A IgG in candidates and donors is generally not recommended as it has no impact on transplant outcomes. However, testing for Hep A IgM is generally indicated as part of the workup for acute hepatitis in HCT candidates because of the increased risk for sinusoidal obstruction syndrome following myeloablative conditioning regimens. Donors who test positive should have the transplant delayed because of a high risk of transmission and increased morbidity and mortality.

Hepatitis B

Hepatitis B, a member of the Hepnaviridae family, is a small DNA virus, which can cause acute self-limited primary infection and resolve or can become a persistent infection

for years or even a lifetime. The virus can be transmitted by percutaneous inoculation via needles, blood transfusion, or contact of mucosal surfaces with blood and other body fluids, including sexual contact. Persistent infection may be accompanied by no hepatic dysfunction, but chronic hepatitis is common and can be associated with a risk for hepatocellular carcinoma. Persistent viremia can pose a risk for transmission by blood transfusion. With routine donor screening for hepatitis surface antigen (HbsAg) and hepatitis B core antibody (anti-HBc), the risk for hepatitis B from a blood transfusion has diminished tremendously. In addition, increasing immunization with hepatitis B vaccines in the community has led to a decrease in the number of patients infected with hepatitis B and hence a decrease in the number of contaminated transfusions. Less than 5% of post-transfusion hepatitis today is caused by hepatitis B.

The overall incidence of hepatitis B has declined from a peak of 11.5 cases per 100,000 in 1985 to 0.9 cases per 100,000 in 2014, with an estimated 850,000 to 2.2 million chronically infected Americans [499, 500]. The prevalence of hepatitis B virus (HBV) is geography dependent. In the United States approximately 0.1–0.5% of the population are hepatitis B carriers [500] compared ≥8% in all of Africa, the Middle East (except Israel), South and Western Pacific islands, the interior Amazon River basin, and certain parts of the Caribbean (Haiti and the Dominican Republic) [501]. Based on these data, the chance of a patient undergoing chemotherapy also being a chronic hepatitis B carrier is increasing. Similarly, the likelihood of a BMT candidate having hepatitis B or being exposed to an HbsAg positive donor is increasing.

Chronic hepatitis B carriers who undergo chemotherapy or BMT have been shown to have an increased risk of liver-related morbidity and mortality [502–512]. At the completion of chemotherapy or BMT conditioning, the patient becomes immunosuppressed. At this time there is an increase in hepatitis B virus replication in the liver, leading to an increased viral load. On recovery postchemotherapy, there is immune reconstitution, which is associated with an acute hepatitis flare. Immune recovery causes an immune-mediated destruction of liver cells containing HBV. The severity of hepatocyte destruction is directly proportional to the viral load in the liver [500, 503, 506]. This results in an acute exacerbation of hepatitis and may progress to fulminant liver failure.

Studies have been performed in lymphoma patients with concomitant HBV undergoing standard chemotherapy. In this population, the development of impaired liver function (as assessed by increased liver function tests) occurred in more than 50% of patients studied. Liver-related mortality was 5%, with reactivation of HBV thought to be the cause of death [506]. Similarly, there are data characterizing an increased risk of HBV morbidity and mortality in HBsAg-positive BMT recipients [513–520].

In the solid tumor population, prediction of who will reactivate HBV is difficult. The most useful marker of reactivation is HBV viral DNA level [495]. Close monitoring of hepatitis markers is essential. Management strategies in the HBV-positive patient undergoing chemotherapy include as the first step removal of those agents likely to potentiate HBV reactivation. Corticosteroids are a common component of chemotherapy regimens and are known to stimulate viral reactivation [521]. If possible, steroid therapy should be avoided, but this may not always be possible. Continuous low-dose corticosteroids have been attempted and are generally no better than pulse steroids [19]. Similarly, daily or weekly schedules have not been shown to be better than schedules calling for administration every third to fourth week. Other drugs that increase the risk for reactivation/exacerbation of hepatitis B include rituximab [213] and alemtuzumab [522].

The alternative approach is to prophylactically administer antiviral agents to prevent HBV reactivation. Interferon has demonstrated efficacy in hepatitis B carriers (but is less effective in Asian hepatitis B carriers). One of the important side effects of interferon is myelosuppression, and for this reason it is often contraindicated in the already myelosuppressed cancer patient. Alternatives include the nucleoside analogues lamivudine and famciclovir. Both of these agents interfere with viral DNA replication, leading to inhibition of serum HBV viral DNA levels, with consequent improvement in liver function tests (LFTs) [523–529].

The most effective treatment strategy is targeting those patients at greatest risk (identified by screening patients for past and current infection with HBV) and administering prophylaxis to prevent viral reactivation [530]. Patients with evidence of disease at the time of chemotherapy, as demonstrated by viral replication (HBV-DNA-positive) should receive an antiviral agent such as lamivudine (100 mg orally daily) or famciclovir (500 mg three times daily). Unfortunately, on many occasions the viral load in the liver is too great to be affected by this treatment. Carriers of HBV can either be treated prophylactically with the nucleoside analogues or closely monitored postchemotherapy for evidence of viral replication. In those patients receiving prophylactic therapy with the nucleoside analogues, therapy should be continued for at least 6 months following completion of chemotherapy [530].

Management of patients undergoing autologous BMT is similar to conventional chemotherapy. Delayed immune reconstitution may lead to a greater risk of reactivation, but the risk is not as great as that seen in allogeneic BMT recipients. Allogeneic BMT in the HBsAg-positive patient is somewhat more complicated. At the time of transplantation, the patient may be actively infected with hepatitis B virus (HBsAg-positive) or alternatively latent HBV may be reactivated following transplantation (HBsAg-negative) [530]. In

the allogeneic setting, the hepatitis B status of the donor must also be considered; otherwise, liver failure secondary to hepatitis B infection is a possible consequence.

The mechanism of viral reactivation is very similar to that seen after chemotherapy. BMT-conditioning chemotherapy causes ablation of both the bone marrow and the immune system, leading to increased viral replication. On recovery of the immune system, an acute flare may occur. Similar flares have been documented when tapering cyclosporin A immunosuppression [502]. 43 hepatitis B virus carriers undergoing autologous or allogeneic BMT with similar preconditioning regimens, none of the autologous BMT patients developed fulminant hepatitis, compared to five allogeneic patients. Impaired LFTs correlated with cyclosporin A withdrawal [502] and could not be controlled by cyclosporin A dose escalation. Potential signs of a hepatitis flare include increases in LFTs, particularly serum aspartate aminotransferase and alanine aminotransferase (ALT). Hepatitis serology should also be monitored. Ominous signs for the development of fulminant hepatitis include changes in serology from HbeAg-negative to HbeAg-positive, the presence of HbcAb IgM, or detection of HBV-DNA [502].

Approaches to the prevention and treatment of HBV in allogeneic BMT patients are complicated and depend on both the donor and recipient hepatitis profiles. Prior to BMT, all patients and donors should have a hepatitis workup, including HBsAg and HBcAb. If the HBsAg is positive, HBeAb, anti-HBe, and HBV DNA (including quantification) should be assessed. Recommendations for management depending on the results of hepatitis screening have been reviewed [134, 531]. The key to management of BMT patients is comprehensive upfront screening and prophylaxis with a nucleoside analogue if infected.

In addition to lamivudine and famciclovir, newer agents, including adefovir dipivoxil, entecavir, telbivudine, and tenofovir, are effective in suppressing viral reactivation [532, 533]. Unfortunately, the emergence of drug-resistant variant viruses with mutations in the HBV polymerase gene has occurred with their use [534–536] and it is unclear whether any of these newer agents should be used for first-line preemptive therapy. An inactivated hepatitis B vaccine is highly protective. In non-immunized exposed individuals, hyperimmune globulin can afford protection. Interferon can suppress hepatitis B replication although the magnitude of benefit is not great and is frequently temporary [537]. In one study an attempt to immunize BMT and leukemia patients against hepatitis B was unsuccessful [538]. In one case persistent hepatitis B was ablated in a patient who received a marrow transplant from a hepatitis B-immune donor [539]. Similar experimental data in mice have also been supportive of the concept that donor immunity can be adoptively transferred to BMT recipients [540].

In conclusion, when managing BMT recipients, consideration should be given to HBV infection in the differential diagnosis in post-BMT patients with abnormal hepatic transaminases not explained by the typical causes of hepatitis, for example, medications, GVHD, or other viral infections (CMV, HSV, VZV). Increases in hepatic transaminases are most likely at the time of immunosuppression tapering. Provided treatment and prophylaxis recommendations and guidelines are adhered to, the risk of developing HBV infection is low.

Hepatitis C

Hepatitis C is a lipid-enveloped single-stranded RNA virus related to members of the Flaviviridae family. Hepatitis C virus (HCV) can be transmitted via blood transfusions, intravenous drug abuse, and needlestick injuries, as well as by vertical transmission. The risk of transmission of HCV by a blood transfusion has decreased significantly following the introduction of screening processes in 1991. The magnitude of the decrease is such that the risk of transmission in donated blood that has passed all screening tests is now 1 in 103,000 [541] compared to 0.19–0.45% prior to 1991 [542]. The seroprevalence of antibody to HCV in most developed countries is approximately 1–2%. As with hepatitis B, there is significant geographical variation in seroprevalence rates, with higher rates of infection in Eastern Europe and Africa than in the United States. The Centers for Disease Control estimates that 2.7–3.9 million people are infected with HCV in the United States [543]. There are six genotypes of HCV, each having a different geographical predominance. The most common genotypes in the United States are 1a and 1b; genotype 1b is also common in Europe. In Asia genotype 6 is most common, in South Africa genotype 5 is most common, in the Middle East and North Africa genotype 4 is most common, and among intravenous drug users the prevalence of genotype 3 is rising. Differences have been seen in the various genotypes in terms of responses to therapy, but there is currently no clear correlation of disease outcome and severity with genotype [544].

Hepatitis C can cause an acute, self-limiting, primary infection and resolve, or it can become a chronic infection. Approximately 85% of HCV-positive patients develop chronic HCV infection (as documented by persistence of HCV RNA in serum) [544]. There are data demonstrating that cirrhosis develops in more than 20% of chronically infected adults an average of 20 years after diagnosis of the acute infection [545]. Like hepatitis B, hepatitis C has been linked to hepatocellular carcinoma. Hepatocellular carcinoma occurs most commonly in those individuals who develop cirrhosis. Other complications seen commonly in HCV-infected people include elevated transaminases; symptoms of terminal liver failure, that is, portal hypertension and hepatic encephalopathy; and hepatic fibrosis. Hepatitis C has

also been reported to cause extrahepatic manifestations, for example, porphyria cutanea tarda, mixed cryoglobulinemia, and glomerulonephritis [546].

Hepatitis C virus infection can be documented serologically by the detection of antibody directed against HCV in serum (anti-HCV) using enzyme immunoassay (EIA) methodology. These tests have been shown to detect anti-HCV in more than 97% of infected individuals. The test is useful for demonstrating past or present infection but is not able to differentiate among acute, chronic, and resolved infection [547]. The EIA technique occasionally produces false positive results, and therefore confirmatory testing with a supplemental assay is often performed. In cancer patients and the immunocompromised, it is prudent to test for HCV RNA, as this population may produce a false negative result due to inability to produce sufficient antibodies for detection by EIA. The virus itself can be detected in blood through the use of reverse transcriptase polymerase chain reaction (RT-PCR) amplification of HCV RNA. This test enables detection of circulating HCV RNA early in the course of HCV infection, that is, 1–2 weeks following exposure. The presence of HCV RNA indicates an active infection. The final test is a quantitative assessment of the concentration of HCV RNA in the blood (viral load assessment). This test is only useful for determining response to therapy and does not correlate with the severity of hepatitis or overall prognosis.

Hepatitis C infection is relatively common in the cancer (prevalence up to 70%) [529, 548–557] and BMT (3–70%) [558–562] population, particularly those who were treated prior to 1991. These patients have been at greater risk of developing HCV infections than the general population because of the large number of blood products they received during or following therapy. Since the introduction of routine screening tests, the proportion of infected patients has decreased. Because of the large number of people infected with hepatitis C worldwide, the chance of a BMT patient or donor being hepatitis C positive is relatively high. Therefore, appropriate diagnostic and subsequent precautionary measures should be taken to avoid fulminant liver failure. Patients thought to be at risk for hepatitis C (i.e., those with increased ALT pretransplantation that is not explained by other likely diagnoses, those with a history of intravenous drug abuse, with a history of blood transfusion prior to 1991, or with multiple sexual partners) should be evaluated for hepatitis C infection by HCV RNA PCR detection technology prior to transplantation.

Patients who are infected with HCV and undergo BMT are at increased risk for multiple hepatic problems in addition to that seen in immunocompetent patients. Sinusodial obstruction syndrome (SOS, previously known as veno-occlusive disease [VOD]) occurred more frequently in patients who were HCV-RNA positive prior to transplant compared to HCV-negative patients (87% vs. 66%, respectively, $p < 0.004$) [562]. Severe SOS was also increased in patients who were HCV positive prior to transplant compared with HCV-negative patients (48% vs. 14%, $p < 0.0001$) [562]. Hepatic inflammation may be seen 3–6 months after transplantation, which is coincident with immune reconstitution and discontinuation of immunosuppressive medications [562]. Patients who have cirrhosis at time of transplantation are at increased risk of developing hepatic decompensation post-HCT [563, 564]. Finally, patients receiving mycophenolate mofetil may develop a firbrosing cholestatic hepatits, which is an aggressive form of viral hepatitis that usually occurs before day 100 [565]. The impact of HCV infection in long term survivors after HCT was initially studied by Ljungman et al. [556]. The diagnosis was based on the HCV positivity either by PCR for HCV RNA or by second-generation ELISA and RIBA or EIA supplemental assay. Of 161 surviving patients transplanted between 1978 and 1991, 28 (17.4%) were found to have chronic HCV infection. No signs of severe progressive liver disease were shown among the patients included in this study with a follow-up median time of 6.1 years [556]. Thomas et al. with an average follow-up of 6 years found no evidence of cirrhosis [566]. In 1999, the Seattle group reported a cohort of 355 patients that underwent HCT between 1987 and 1988 from which 113 (32%) were HCV RNA-positive by day 100 posttransplant. During 10 years of follow-up, no patients developed clinical evidence of liver disease, and HCV infection did not impact the actuarial survival of long-term survivors over this time period [562]. It was thus concluded that HCV infection was not associated with excess mortality over 10 years of follow-up. However, the same group observed the development of cirrhosis leading to hepatic decompensation and hepatocellular carcinoma in HCV-infected, marrow transplant recipients, surviving beyond 10 years. Among 3721 patients who survived 1 or more years after HCT, 31 developed cirrhosis. Cirrhosis was attributed to HCV in 15 of 16 patients presenting more than 10 years after HCT [567]. HCV infection ranked third, behind infection and GVHD, as a cause of late death [568]. Moreover, cirrhosis was diagnosed beyond 10 years after transplantation in 13 of 15 patients and 3 cases of hepatocellular carcinoma was observed in this cohort. Ivantes et al. also found cirrhosis on a smaller group of patients followed up more than 10 years after HCT [569]. Thus, HCT recipients with HCV infection present a higher risk of earlier cirrhosis.

The presence of HCV is not an absolute contraindication to transplantation. In general, the course of chronic liver disease is insidious, and progress is slow for at least 10–20 years. The determination of whether transplantation should proceed and of when to transplant is dependent on the degree of underlying liver dysfunction, which should be established prior to transplantation. It is recommended that all candidates for HCT with HCV infection must be assessed for evi-

dence of chronic liver disease [134]. Liver biopsy is advised in the setting of associated iron overload, history of excessive alcohol intake, history of hepatitis C > 15 years, and clinical evidence of chronic liver disease [530]. Patients with evidence of cirrhosis should avoid conventional Cy or TBI containing conditioning regimens as they are associated with a 9.6-fold increase in fatal sinusoidal obstruction syndrome [562]. However, even with reduced intensity conditioning regimens, there is a significant mortality risk for patients with cirrhosis [570].

In the allogeneic BMT setting, the HCV status of the donor must also be evaluated. Hepatitis C virus transmission to recipients of HCV RNA-positive allogeneic and syngeneic marrow products [571] occurs universally. Within days of marrow reinfusion, patients develop viremia, although evidence of clinical and biochemical hepatitis may take weeks to months to develop. Damage to hepatocytes in HCV infection is mediated predominantly by HCV-specific cytotoxic T cells, and therefore biochemical abnormalities will only become apparent on engraftment and reconstitution of cell-mediated immunity [562, 563, 572]. If time permits, the best action is to treat the donor in an attempt to render the donor's serum HCV RNA undetectable at transplantation. However, treatment of HCV infection may take 6–12 months, time which is not always available in transplantation because of the underlying nature of the malignant disease. Again, donor HCV seropositivity is not a contraindication to transplantation, priority being allocated to an HLA match, whether donor and recipient are HCV-positive or -negative.

Historically, the standard HCV treatment for the general population was interferon-α, either alone [573–579] or in combination with ribavirin [580–582]. Interferon (3 million units subcutaneously three times per week for 12 months) is active and has been shown to reduce the level of HCV viremia and improve hepatic transaminase abnormalities [583–585]. Long-term remissions can occasionally be seen (in 15–25% of cases) [547]. Unfortunately more than 50% of patients who respond initially relapse after treatment cessation. Predictors of a poor response to interferon include a high HCV RNA titer and HCV genotype 1 (the most common genotype in the United States). The combination of ribavirin (1000 mg/day divided into two doses for patients weighing less than 75 kg; 1200 mg/day divided into two doses for those over 75 kg) and interferon has significantly improved response rates compared to monotherapy (36% vs. 18%, $p < 0.047$) [581], and the responses were sustained over 12 months. In most clinical situations, combination therapy is preferred. The combination of pegylated alpha interferon and ribavirin produced a sustained virological response rate of approximately 55% [583, 584]. Responses were better in genotype 2 or 3 infections versus genotype 1 infections (approximately 80% response with a 24-week treatment duration vs. 50% with a 48-week duration, respectively). There is concern about the use of combination therapy in allogeneic BMT patients as interferon is associated with an increased risk of GvHD and mortality within 6 months of transplantation [585–587], and ribavirin monotherapy has been shown to be ineffective in the general population [588–590], although there is one small case series that demonstrates efficacy in BMT patients [591]. However, a large cohort study showed no increased risk of GVHD among allogeneic HCT recipients treated with interferon with or without ribavirin [592]

Direct acting antivral (DAA) agents are oral agents that target various HCV-encoded proteins vital to the replication of the virus (Table 51.7). When used in combination in normal individuals, they have demonstrated favorable safety profiles and have demonstrated excellent sustained viral repression with the possibility of cure of HCV. Unfortunately, the efficacy and safety of DAAs have not been extensively studied in infected HCV recipients. Current guidelines by the ASBMT are extrapolated from recommendations by the American Association for the Study of Liver Diseases (AASLD) and Infectious Disease Society of America (IDSA) [593].

One of the issues that has yet to be addressed in a large study is how to manage the long-term survivor of cancer or bone marrow transplantation. As supportive care technologies improve, the length of overall survival is increasing, which will lead to a population of patients with the potential to develop cirrhosis. As mentioned, blood transfusion products were not screened for HCV prior to 1991. With the insidious nature of the disease, the implications of cancer treatment and transplantation in this group may not have been truly realized before mid-2000. Common sense tells us that those patients developing signs of cirrhosis and liver failure should be treated in the same manner as the general HCV-infected population. Currently, routine monitoring of HCV-RNA is

Table 51.7 Direct acting antiviral (DAA) agents for hepatitis C

Direct acting antiviral (DAA) agents
Protease inhibitors
Boceprevir
Paritaprevir
Simeprevir
Telaprevir
Ritonavir
NS5A inhibitor
Daclatasvir
Ledipasvir
Ombitasvir
Nonnucleoside polymerase inhibitor
Dasabuvir
Nucleoside polymerase inhibitor
Sofosbuvir
Nucleoside analogue
Ribavirin

not advised in HCT recipients with chronic HCV infection. However, viral load monitoring should be considered in patients who have an unexplained elevation in ALT or are receiving active therapy for HCV. In addition, all long-term survivors of cancer with chronic viral hepatitis should be offered vaccination because of the increased risk of fulminant hepatitis should they develop acute hepatitis A [594, 595].

Hepatitis G

Hepatitis G virus (HGV) is a RNA virus that belongs to the Flaviviridae family [596, 597] and is closely related to the GB virus-C (GBV-C) [598]. It prevails worldwide, and the viral RNA has been detected in 1–5% of volunteer blood donors [598, 599]. The prevalence of HGV in patients undergoing BMT ranges from 18 to 61% [600–605]. Following BMT, immunosuppressed patients have been reported to have an increased risk of HGV infection after transfusion-related exposure [601, 606]. This has not translated into accelerated HGV-RNA replication [605].

Currently, the clinical relevance of HGV infection and its long-term effect on BMT patients remain unresolved. HGV has not been shown to affect engraftment kinetics, which indicates no direct effect of HGV on hematopoietic progenitors [607]. The data on the effect of HGV on liver function are conflicting. There are data demonstrating no effect of HGV on the clinical and biological diagnosis of hepatitis in immunosuppressed patients in several studies [604, 606]. However, there are also data in pediatric patients demonstrating increased ALT levels in the absence of other known causes [600]. The maximum ALT levels were statistically higher than those of uninfected patients, although the mean values of ALT showed no difference. This suggests that the effect of HGV on ALT is transient and self-limiting [600].

At present blood for transfusion is not routinely screened for HGV, which is not considered to be a serious pathogen nor a significant cause of hepatitis. Clearly, additional large studies of HGV-infected immunocompromised patients are needed to definitively characterize the effect of HGV on engraftment and hepatotoxicity.

Other Viruses

Papovaviruses

Infection by JC and BK viruses occur early in childhood and are widespread. In most individuals it is asymptomatic, but the viruses persist in the kidney and urogenital epithelium. Asymptomatic virus excretion increases in immunocompromised patients [608, 609]. The JC virus has been associated with progressive multifocal leukoencephalopathy and has been described in patients with chronic lymphocytic leukemia or lymphoma and following BMT [610–612]. Dissemination with involvement of multiple visceral organs, including the lungs, has also been noted [608]. The BK virus has been associated with hemorrhagic cystitis, especially in allogeneic BMT patients, in whom the risk is three times as high as in similarly treated autologous BMT recipients [613–617]. Occasionally adenovirus type 11 can also be associated with hemorrhagic cystitis [614]. Both humoral and lymphoproliferative responses to active viral shedding have been noted, but whether these are immunoprotective remains uncertain [618]. At present, there is no known effective therapy, although cases of response to vidarabine, ribavirin, ciprofloxin, or cidofovir have been reported.

Parvoviruses

Human parvovirus B19 is a small DNA virus, which causes erythema infections during childhood. It is an occasional cause of arthritis and in patients with chronic hemolytic anemia can be a cause of a transient aplastic crisis. It is a potential cause of pancytopenia, rash, or chronic anemia in immunocompromised patients, including leukemia and BMT patients [619–627].

Intranuclear lesions within erythroid precursors in bone marrow specimens have been noted in acutely infected patients [628].

Marrow recipients with GVHD may have chronic infection. The infection can be documented by detection of virus-specific antibody against the B19 antigen by ELISA or of viral DNA by PCR. The virus has been detected in respiratory secretions, bone marrow, and blood. There is no specific antiviral treatment, but reducing the intensity of chemotherapy can be helpful [623], and immune globulin may also be of benefit.

Retroviruses

Human immunodeficiency virus is a retrovirus that is the causative agent of AIDS. Sexual transmission and transmission via blood transfusion or organ transplantation has been well documented. During the early 1980s, prior to the development of a screening serologic test for blood products, patients with hematologic malignancies receiving blood product support were susceptible for contracting HIV infection. Subsequent to 1985 with routine screening, the risk of transmission via a blood product is 1 in 500,000 or lower. Several cases of AIDS in patients with hematologic malignancies have been documented [629]. It is uncertain that the course of illness in immunocompromised patients is substantially different than in nonimmunocompromised patients.

The association of HIV infection with Kaposi's sarcoma and aggressive non-Hodgkin's lymphoma has been noted, especially among those with low CD4 counts for more than 1 year [630–633]. There has been limited experience performing bone marrow transplantation in patients with HIV-associated malignancies [634–636]. In some patients, cytoreductive therapy to ablate all lymphohematopoietic-derived cells (the reservoir of latently infected cells) combined with antiviral therapy has had a marked antiviral effect, whereas in others in whom the virus is not very susceptible, the effect has been marginal. Most of the beneficial effects have been transient, and generally patients have succumbed from transplant-related complications or opportunistic infection. With the development of better antiviral agents and the engineering of donor marrow stem cells with genes that confer resistance to viral infection, this might be more promising in future investigations.

In the era of highly active antiretroviral therapy (HAART), there has been a drop in the rates of opportunistic infection and Kaposi's sarcoma. Of interest, however, is that there has not been a concomitant fall in the incidence of AIDS-related lymphomas, at least so far [637–640], with the exception of a decline in central nervous system lymphomas [630, 631]. Unfortunately, most cases present at an advanced stage and have a very poor prognosis, which also has not changed in the era of HAART [637, 641]. In a subset of patients with CD4 counts above 100/ml, higher response rates and longer survival have been noted [642]. Since HAART therapy may preserve immune function for long intervals, diagnosis of lymphomas at an earlier stage may in the future lead to improved treatment outcomes.

Currently, the presence of HIV in patients with a malignancy treated by transplantation does not automatically exclude the patient from this potentially life-saving procedure. In patients with controlled HIV disease, autologous transplantation is a reasonable, with transplant-related mortality similar to non-HIV-infected patients [643–647]. The data for allogeneic transplant, however, is limited although it is feasible [648]. Management of these patients should be coordinated with a HIV specialist and preferably on a clinical trial.

Human T-cell lymphotropic viruses (HTLV)-1 and -2 are type C oncoviruses present in widely scattered populations, including those of the islands of southwestern Japan and the Caribbean basin, where infection is endemic. Screening of blood donors in the United States indicates a small but detectable seroprevalence. Transmission is by blood transfusion, sexual contact, and in utero. Although most infections are asymptomatic, HTLV-1 is the etiologic agent of adult T-cell leukemia/lymphoma [649–651]. There have also been associations between HTLV-2 and malignancy [651], but its role in pathogenesis is uncertain. Adult T-cell leukemia/lymphoma occurs in only a small minority of HTLV-1 carriers. The lifetime risk is estimated to be between 2 and 5% [649].

West Nile Virus

West Nile virus is a flavivirus first detected in the northeastern United States in 1999 and since then has caused over 2.5 million cases of infection in outbreaks of infection in the late summer and early fall throughout the Unites States [652, 653]. In humans, most infections are asymptomatic. However, 20% of infected patients develop West Nile fever, manifested by malaise, anorexia, nausea, myalgias, headache, and occasionally lymphadenopathy [654]. One in 150 symptomatic patients develop meningitis or encephalitis, presenting as photophobia, phonophobia, and meningismus [655]. Patients with encephalitis develop altered mental status, cranial nerve palsies, seizures, and movement disorders. A minority of patients abruptly develop asymmetric weakness that may progress to flaccid paralysis associated with hyporeflexia or areflexia, mimicking poliomyelitis [653, 656, 657]. Acute neuromuscular respiratory failure may develop, with a mortality rate of more than 50% [655]. Hemorrhagic fever has been described as well, similar to syndromes seen with other flaviviruses [658].

Diagnosis of West Nile virus infection in immunocompetent hosts may be made serologically or by reverse transcriptase PCR. An IgM antibody capture assay is available and becomes positive in CSF 3–5 days after onset of symptoms in nonimmunosuppressed hosts [659, 660], before serum antibody develops; CSF IgG is seen approximately 5 days later. Antibody presence may be confirmed with viral neutralization studies. IgM antibodies may persist in serum for up to 12 months after infection resolution, and IgG may persist for years. Immunocompromised patients demonstrate delayed seroconversion, making diagnosis of acute infection difficult at times. Nucleic acid testing in plasma or CSF is the most useful diagnostic test in this setting [661].

Several cases of West Nile virus infection have been reported in recipients of BMT [659, 662]. In most well-described cases, infection occurred 3–5 months after engraftment while on calcineurin-based prophylaxis against or treatment for chronic GVHD. Fever, lethargy, and progressive bilateral extremity weakness were noted. Hyporeflexia or areflexia were present, and CSF contained 0–6 white blood cells/μL. Serologic studies were negative in CSF and blood in most cases (including IgM assays). Diagnosis of West Nile virus infection was made by PCR performed on serum and CSF. All of the described patients died.

There are no antiviral agents that have proven efficacy in the treatment of West Nile virus infection. Ribavirin possesses in vitro activity but demonstrates poor clinical efficacy [663, 664]. Intravenous immunoglobulin with high titers of anti-West Nile virus antibodies were demonstrated to have significant clinical benefit in animal models. However, immunoglobulins derived from US donors has been ineffective in treating acute infection [652, 665, 666]. Overall mor-

tality from West Nile virus are 4–20% with significantly higher rates in immunosuppressed patients [655, 667].

Summary

The clinical significance of viral infections in patients with hematological malignancies has assumed greater importance during the last two decades. In part, this is related to more frequent recognition of viral syndromes due to improved techniques of documenting various viral pathogens by more accurate and more accessible diagnostic assays. It is also attributable to increasing use of dose-intensive or immunosuppressive treatment regimens, which have greater attendant immunosuppression and susceptibility for viral infection. With increasing awareness of the substantial morbidity due to these viral pathogens, it has become extremely important to recognize these infections. With the expanding array of antiviral agents, vaccines, and immune modulators, it has now become possible to intervene therapeutically to prevent infection, reduce morbidity, or prevent death from these pathogens. With continuing investigations it is likely that a number of unexplained syndromes encountered in patients with hematologic malignancies will be identified in the future as having a viral etiology. These advances hopefully will further improve our supportive care in this patient population and permit further strides in the control of hematologic malignancy.

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52 Therapeutic Cytapheresis, Plasmapheresis, and Plasma Exchange in Neoplastic Diseases of the Blood

Janice P. Dutcher

Introduction

Apheresis is defined as the process of removing a specific component of blood and returning the rest of the blood to the donor [1–3]. This is an automated procedure in the case of donors, and allows the collection of greater amounts of the product than could be prepared from removal of a single unit of blood with centrifugation into its components in the laboratory. The apheresis process is currently utilized extensively for collection of platelets, plasma, and less frequently, granulocytes. One donation can amount to a full transfusion, equivalent to the number of platelets, for example, derived from 6 units of whole blood [1–3].

The concept of therapeutic apheresis utilizes the process of automated collection of whole blood or components thereof to improve/reverse/temporize a potentially dangerous clinical situation by removal of blood or blood components that are considered harmful or excessive in amount [4, 5]. The component may be whole blood in the case of symptomatic polycythemia, or leukapheresis or plateletpheresis in situations of dangerous excesses of white blood cells (WBC) or platelets. Plasmapheresis is conducted to remove components of plasma that are contributing to disease. In hematologic malignancies, this is most frequently a clonal immunoglobulin, in the setting of symptomatic hyperviscosity or cryoglobulinemia. Apheresis/red cell exchange has an additional role in certain situations in sickle cell anemia patients with organ- or life-threatening crisis, or in the setting of prior stroke as prevention. Plasmapheresis has an additional role in removal of immunoglobulins in paraproteinemic polyneuropathies, but beyond these entities, most plasmapheresis is done for nonmalignant diseases. Plasmapheresis has also been explored for antibody removal in organ transplantation. There are varying levels of evidence to support its role in nonmalignant diseases, but the clinical scenarios described earlier are supported by clinical evidence in the medical literature [4, 5]. This chapter will focus on the evidence for therapeutic apheresis in neoplastic/hematologic diseases. As treatment for the various hematologic malignancies has improved, the frequency of use of therapeutic apheresis has decreased, but it remains an important therapeutic option in emergent situations, as will be discussed.

The successful application of apheresis has been greatly enhanced over the past 30–50 years by remarkable technological developments. Initial developments, in the 1950s, demonstrated that blood components could be separated from one another in the laboratory by techniques of differential centrifugation, with the heavier components (red blood cells and granulocytes) in the lower levels and platelets and plasma above [6, 7]. Subsequent technology utilized an intermittent technique where, as blood was collected in a conical bowl (Latham bowl), the blood components separated into layers with centrifugation and were visualized over the bowl curvature, with red blood cells (RBCs) at the bottom and plasma on top, and this procedure allowed removal of plasma and could be used to wash red cells. Once the collection was completed, the remainder in the bowl contents was returned to the donor, and the cycle was then repeated. This approach was eventually utilized to separate out and collect platelets and plasma with the return of the red cells and some plasma to the donor. This process was repeated for 6–10 times over the course of 2–4 h to collect sufficient platelets for a transfusion [8]. Simultaneously, the concept of continuous flow centrifugation was evaluated and implemented in studies at the National Cancer Institute in a collaboration between Dr. Emil Freireich and an engineer from IBM, George Judson, whose son was a patient. They developed a prototype machine that allowed collection of excess leukemia cells with continuous return of plasma and red cells to the subject [9]. The continuous flow approach and collection techniques have been refined with numerous improvements in the technology over the past 30 years, which have led to efficient donor collection techniques, and

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safer therapeutic collections [5, 10, 11, 12]. While manual collections are still feasible, the majority of therapeutic procedures are conducted using automated cell separators. The notable exception is the use of manual phlebotomy in polycythemia.

Complications of apheresis are related to the procedure itself, including the venipuncture, or placement of a central venous line in the case of high volume plasmapheresis, with occasional problems of venous access (pseudoaneurysm, bleeding, and clotting). All apheresis procedures require that the blood being processed be anticoagulated to avoid clotting in the system, usually with citrate solutions, either acid citrate dextrose (ACD) or citrate-phosphated dextrose (CPD), both of which work by binding calcium. Occasionally in the procedure, with the return of citrated plasma, symptoms of hypocalcemia can develop, including at a minimum circumoral paresthesias, but more seriously irritability or rarely tetany [13]. These symptoms can be ameliorated with oral calcium carbonate lozenges and with slowing down the rate of flow of the procedure. The use of these anticoagulants does not cause systemic anticoagulation in the donor or subject.

Hydroxyethyl starch is used in granulocyte collections, to rouleaux RBCs and allow separation of granulocytes from the red cell layer. Although no long-term toxicities have been noted, acute reactions such as volume expansion and hypersensitivity have been reported [14, 15].

Another side effect that can occur is hypotension due to loss of volume, either from the extracorporeal volume removed in the apheresis tubing and system, or as a result of removal of the components. For therapeutic cytapheresis in patients with neoplastic diseases, some subjects may start with anemia and this can be worsened by the infusion of additional return fluids with hemodilution. Such patients may become symptomatic during or after the procedure.

Cytapheresis

Leukapheresis/Hyperleukocytosis

Acute and Chronic Myeloid Leukemia

Historically, hyperleukocytosis was not an infrequent presentation of acute myeloid leukemia (AML), blastic crisis of chronic myeloid leukemia (CML-BC), and less commonly, the initial presentation of chronic phase of CML. Hyperleukocytosis is defined as a WBC count of greater than 100,000/μL and in acute leukemia, this can be composed almost exclusively of blast cells. The effect of elevated WBC on blood viscosity is a function of the size and shape of the cell involved, causing stasis in the microvascular bed or forming cellular aggregates [16–18]. As the blast cells divide in situ, they may rupture fragile capillary vessels, leading to hemorrhage. These multiple effects of hyperleukocytosis are particularly dangerous in the capillary beds of the lungs and brain, and can lead to early death [19–23]. Metabolic effects from the cells utilizing oxygen in situ can lead to organ hypoxia as well.

Blast cells are the least deformable WBC, so a high WBC count consisting of mostly blasts is the most dangerous situation, and must be rapidly reduced to avoid serious hemorrhagic complications [16–18]. Patients may also have nonhemorrhagic symptoms such as impaired mental status, headache, or respiratory distress. In patients with significant hyperleukocytosis, or those needing a rapid reversal of symptoms, or in whom there are delays in administering systemic therapy, leukapheresis should be considered.

There is a three decade long series of reports demonstrating reduction in blast cell counts with leukapheresis, with improvement of symptoms and the ability to proceed with antileukemic therapy. Several groups have looked specifically at the effect of leukapheresis on early mortality (within the first 7–21 days of treatment) and on long-term survival in patients with leukemia, presenting with very high WBC counts [24–26] (Table 52.1). In a French study, all 53 patients with an initial WBC count of greater than 100,000/μL (median 160,000/μL) underwent leukapheresis, which significantly reduced the WBC count to less than 100,000/μL in 32 patients (60%) and the median WBC at start of chemotherapy for all patients was 85,000/μL [24]. The early death rate was 2 of 53 patients, which is lower than previously published data in such a group of patients [24, 27]. A retrospective study from M.D. Anderson Cancer Center evaluated 146 patients with an initial WBC count of greater than 50,000/ul. 71 of these patients underwent prechemotherapy leukapheresis directed by the treating physi-

Table 52.1 Leukapheresis in AML presenting with hyperleukocytosis

Ref.	No. of patients	Pre-apheresis	Patients	Early death
		Minimum WBC	No. of apheresis	
		Median WBC	No. of no apheresis	Within 7–21 days
Thiebaut et al. [24]	53	100,000/μL	53	2 (4%) within 1st 21 days
		160,000/μL	0	
Giles et al. [25]	146	50,000/μL	71	13%
			75	23%
				Within 1st 14 days
				In multivariate analysis, $p = 0.006$
Bug et al. [26]	53	100,000/μL	25	16%
			28	32%
				Within 1st 21 days

cian and 75 did not [25]. They found a reduction in initial 2-week mortality, 13% vs. 23%, for those who underwent initial apheresis, compared to those who did not. There was no evidence of effect on longer term or overall survival [25]. Similarly, Bug et al. evaluated 53 patients who presented with a WBC count of greater than 100,000/μL, among whom 28 received chemotherapy without leukapheresis and 25 underwent leukapheresis prior to chemotherapy [26]. By day 21, there was a 25% mortality overall. However, there was significantly less mortality among the cohort who underwent leukapheresis compared to those who did not undergo leukapheresis (16% vs. 32%, $p = 0.005$). Again, the overall survival was similar in both groups and apheresis did not impact on the long-term outcome [26]. As has been noted previously, patients with hyperleukocytosis and hyperproliferative AML have a worse long-term prognosis even with a similar remission rate compared with patients presenting with lower WBC counts [27].

These were all retrospective evaluations, and it is therefore difficult to account fully for other biological factors contributing to early death, or to account for factors leading to the initiation of apheresis. Nevertheless, there does appear to be consistency in reducing the risk of early death and allowing these patients to proceed with antileukemic therapy. Single institution series in the era of modern antileukemic therapy continue to recommend initial leukapheresis for symptomatic hyperleukocytosis to allow subsequent definitive systemic treatment, at the same time suggesting that improved supportive care and chemotherapy regimens may reduce the necessity of leukapheresis [28, 29].

In the initial phase of evaluation, it is usually advisable to not administer red blood cell transfusions until the WBC count is lowered to avoid exacerbation of elevated viscosity [30].

Of interest, a meta-analysis review of 20 studies from 1980 to 2012 utilizing leukapheresis for patients with AML and hyperleukocytosis was recently published [31]. This reflects treatment of 1500 patients from 1975 to 2012, and clearly there has been an evolution over time of induction chemotherapy and rapid initiation of treatment and supportive care including urate oxidase. Nevertheless, the interesting finding is that no change in early mortality has been noted over the course of three decades of early intervention for hyperleukocytosis, either with leukapheresis or low dose chemotherapy/hydroxyurea. This analysis likely reflects the actual biology of the disease of these patients, with limited ability to actually change the course. These authors call into question the continued use of initial leukapheresis with the current ability to rapidly initiate antileukemic therapy and supportive care. At the same time, they discuss the limitations of such a report, in terms of confounding bias and retrospective observations with selection bias. Nevertheless this work requires additional discussion and evaluation of an approach that has been accepted for three decades.

Recent guidelines from a working committee of the American Society for Apheresis continue to list hyperleukocytosis with symptoms of leukostasis as a level 1 indication for leukapheresis, but it is advised that the decision to employ leukapheresis should be individualized in the asymptomatic high WBC count setting [5]. Thus, therapeutic leukapheresis is considered an appropriate intervention in patients with myeloid leukemias presenting with extremely high WBC counts. This is done in conjunction with systemic therapy, and usually achieves a rapid reduction in WBC count. It is rarely an ongoing process, since systemic chemotherapy will continue to further reduce the WBC.

Acute Lymphocytic Leukemia

Hyperleukocytosis can occur in patients with acute lymphoblastic leukemia, although less commonly than in myeloid leukemias, and the symptoms are less likely to be severe, since the rheology of the lymphoblasts is such that they are more deformable than myeloid blasts. Therefore, symptoms may not occur until there is an extremely high WBC count in acute lymphocytic leukemia. Again, leukapheresis can produce a rapid reduction in WBC count, but should be accomplished in conjunction with the rapid initiation of systemic therapy. Early studies suggested benefit of this approach in reducing early complications [32, 33]. Prior to effective systemic therapy, studies in children utilized multiple leukapheresis procedures to attempt to "debulk" the disease [32, 33]. More recently, the role of leukapheresis is being called into question in childhood ALL in the era of rapid onset of induction therapy and urate oxidase and other means of supportive care [34].

Sezary Syndrome (Cutaneous T-Cell Lymphoma)

The Sezary syndrome (SS), usually associated with the erythrodermal variant of cutaneous T-cell lymphoma (CTCL), reflects the presence of circulating malignant CD4+ T cells, with characteristic clonal immunophenotypes. These circulating clones are the same as the T-cell clones noted in the cutaneous lymphocytic infiltrates. Given the known sensitivity of cutaneous involvement of CTCL to psoralen and ultraviolet irradiation [35–37], Edelson and colleagues devised the approach of extracorporeal photopheresis (ECP) which is currently utilized particularly in the setting of Sezary syndrome [38]. In this approach, peripheral blood lymphocytes are removed by apheresis, exposed to a photosensitizing agent, and then exposed to ultraviolet A radiation in an extracorporeal circuit, and then returned to the patients [37, 38]. The original system was developed by Therakos, and since that time, additional systems and modifications of the original have occurred over the past 30 years, increasing efficiency, adapting to venous

access needs, and to allow administration to pediatric patients for graft-versus-host disease (see below) [39]. Although a number of systemic agents are available for CTCL, these are usually reserved for more advanced disease, and consensus guidelines in the USA and Europe recommend the utilization of ECP in the erythrodermal/SS stage of disease without bulky nodal involvement, and with the presence of a circulating T-cell clone [37, 39–42]. In carefully selected patients, durable responses to ECP are not uncommon [39]. Systemic therapy has been combined with ECP including bexarotene, interferon-alpha, low dose methotrexate, and, most recently, the monoclonal antibody, alemtuzumab, directed against CD52 [43, 44]. Alemtuzumab appears to be more effective in this entity and current trials are combining this agent with ECP [45, 46]. Histone deacetylase inhibitors have also been approved for relapsed CTCL and are used second-line in SS [37]. Multiple mechanisms have been theorized for the effectiveness of ECP, most notably the induction of apoptosis of the malignant cells, with antigen presentation initiating an immune response against the damaged CD4+ cells in vivo, cytokine production (both pro-and anti-inflammatory), and generation of regulatory T-cells [39, 47, 48]. The appearance of these immunological alterations has led to the use of ECP in chronic graft-versus-host disease (see below). In centers treating CTCL, the majority find a role for ECP in SS/erythroderma that leads to quality skin responses and clinical benefit [5, 37, 39, 42].

Graft-Versus-Host Disease

For more than 20 years, the immunomodulatory technique of extracorporeal photopheresis has been utilized and evaluated in chronic graft-versus-host disease (cGVHD) and it is more commonly being utilized in acute GVHD as well [5, 39, 42]. As experience with the technique and a better understanding of the immunomodulatory effects have developed, there has been a greater interest in utilizing this approach. Whereas early reports described treatment of patients with refractory, steroid-resistant cGVHD [49–52], its use is widely recognized as a second-line approach to cGVHD, allowing rapid tapering of steroids even with the advent of newer immunosuppressive therapies, and its safety profile is excellent [39, 42]. In 2008, Flowers et al. reported the first randomized prospective trial conducted at multiple centers, designed to evaluate the addition of ECP to conventional treatment in terms of total skin score (TSS) after 12 weeks of treatment [53]. Among the 95 patients enrolled, greater than 90% in each arm had extensive cGVHD and half of the patients had been on corticosteroids for more than 1 year. In this randomized trial, there was no statistical difference in TSS at week 12 (15% improvement for ECP containing arm vs. 9% improvement in control arm). However, there were differences in secondary endpoints, suggesting an additive beneficial effect. At week 12, 25% of patients who received ECP in addition to conventional therapy had a >50% reduction in total daily dose of corticosteroids compared to 12.8% of those on conventional therapy alone. Also at week 12, the percentage of patients with a 50% or greater reduction in dose of steroids and at 25% or greater improvement in TSS was higher for the ECP group, 8.3% vs. 0% for controls. Extracutaneous cGVHD in other organ systems that showed greater benefit from the addition of ECP included the eye (30% vs. 7%, $p = 0.04$), oral mucosa (53% vs. 27%, $p = 0.06$), and joints (22% vs. 12%, $p = 0.06$) [53]. In the subsequent decade, numerous studies have been reported, confirming response in cutaneous cGVHD, including complete responses, as well as in those patients with hepatic and mucosal involvement, and two randomized multicenter trials are ongoing [39, 54, 55].

Current apheresis guidelines from Europe and the United States list ECP for steroid-refractory cGVHD [5, 39, 42]. The North American guidelines delineate that the best evidence of response is for cutaneous cGVHD, with acute skin GVHD and non-skin GVHD as lesser recommendations [5]. Immunomodulatory events that have been noted include apoptosis of lymphocytes, with antigen presentation, modulation of dendritic cells, a switch to anti-inflammatory cytokines, and apparent activation of regulatory T-cells to modulate the prior immune response [56–59].

Chronic Lymphocytic Leukemia

Historically, chronic lymphocytic leukemia (CLL) was an appealing target of therapeutic leukapheresis in view of the slow accumulation of mature lymphocytes, and with disease progression defined by accumulation of such cells [60–62]. Essentially, a process of debulking by cellular removal could lead to reductions in WBC count, hepatosplenomegaly, improvement in marrow function, and modest clinical improvement [60, 62]. Additionally, in patients whose disease led to hemolytic anemia with rising WBC counts, leukapheresis has been used to control this complication of the disease by controlling the WBC count. More recently, with the use of rituximab in CLL, occasional reports utilize leukapheresis to reduce the WBC count below 25,000/μL prior to initiating rituximab therapy, to avoid complications when rituximab is administered to patients with high lymphocyte counts. For the most part, symptoms of hyperleukocytosis are exceedingly rare in CLL, given the small size and low viscosity of the cells, and symptoms of leukostasis have only rarely been reported, but at WBC counts in excess of 500,000/μL [63].

There is rarely an indication for leukapheresis in CLL, even with the potential for "debulking." The possible exceptions may be to reduce the WBC count to control hemolysis, should systemic therapy be contraindicated (i.e., contraindication to

or poor tolerance of corticosteroids), and if rapid WBC count reduction is deemed necessary prior to administration of rituximab.

Plateletpheresis/Thrombocytosis

Thrombocytosis is usually an incidental laboratory finding, defined as a platelet count > 450,000/μL. However, platelet counts as high as 1,000,000/μL may be noted in asymptomatic patients. When discovered, differentiation between a reactive thrombocytosis (and the search for other medical diagnoses) versus a clonal myeloproliferative disorder must be sought [64]. The most common causes of reactive thrombocytosis include bleeding, iron deficiency, or inflammatory conditions, including malignancy and autoimmune disorders. Increased normal platelets almost never predispose to thrombosis or vascular complications. However, thrombocytosis associated with myeloproliferative disorders, such as essential thrombocytosis, CML, and polycythemia vera, is often associated with vascular events, including thrombosis and bleeding [59, 60]. Abnormalities of platelet function have also been described in clonal thrombocytosis, possibly contributing to these adverse events [64, 65].

The need to lower the platelet count by pharmacologic measures depends on the risk of bleeding or thrombosis or whether such events have already occurred. Patients with cardiovascular risk factors, age greater than 60 years, or a history of bleeding or thrombosis are at increased risk, and their platelet counts should be controlled with medication and aspirin, if able to tolerate aspirin [64]. Asymptomatic patients with no risk factors remain a dilemma, whether to treat and when [66].

The role of plateletpheresis is relatively limited in clonal thrombocytosis, and has been primarily utilized in patients with acute thrombotic symptoms, such as microvascular ischemic complications that are unresponsive to antiplatelet agents [67–69]. Additionally, there are reports describing prophylactic plateletpheresis in patients at higher risk categories during periods of stress, such as pregnancy [70]. Plateletpheresis is a consideration in other acute clinical situations at risk for thrombosis such as emergency surgery, since this approach provides the most efficient and rapid means of reducing the platelet count, far more rapidly than with medication alone [71, 72].

Erythrocytapheresis (Red Cell Exchange)

In contrast to the management of hyperleukocytosis or thrombocytosis in patients with clonal disorders, when it comes to removal of red blood cells in the management of clonal erythrocytosis (polycythemia vera), the recommendation is to utilize manual phlebotomy, 1 or 2 units at a time. There is no need for an automated procedure with a blood cell separator since the goal is just removal of whole blood and there is no need for a continuous flow approach.

However, red cell exchange, either manually or using a blood cell separator, is recommended in selected cases of sickle cell anemia crises [73]. The hypoxic sickled blood has a higher viscosity than unsickled SS blood, and viscosity is much higher than that of normal hemoglobin A blood. The combination of increased viscosity in small vessel beds and decreased oxygen in sickled red blood cells leads to tissue hypoxia and damage. The goal of an exchange of red cells is to both remove the hemoglobin S cells and infuse red cells with hemoglobin A and normal oxygen carrying capacity. Many patients with SS disease are maintained with simple transfusion or partial manual exchange to maintain a low hemoglobin S percentage of cells to prevent complications, such as recurrences of acute chest syndrome, or frequent pain crises which limit normal life function. Additionally, prophylactic transfusion has been adopted as a preventative measure to decrease the incidence of stroke in SS children with abnormal transcranial Doppler studies [73–76].

However, in serious acute situations, such as acute stroke or severe acute chest syndrome not responsive to simple red cell transfusion, a more aggressive apheresis approach is often indicated [73, 77, 78]. Since acute chest syndrome is the leading cause of death among patients with SS disease, diagnosis and management are critical [77]. Automated exchange is more efficient and there is rapid removal of sickled blood with simultaneous replacement with hemoglobin A red cells. There is often a very rapid clinical benefit. Currently, red cell exchange is considered appropriate in the setting of acute stroke, acute chest syndrome, and as prophylaxis for primary or secondary stroke [73, 77, 78]. Although exchange transfusion is occasionally done in the setting of multiorgan failure and for acute priapism, there are less data demonstrating benefit.

Stem Cell Collection

Over the past 15 years, leukapheresis collection of peripheral blood stem cells from stimulated donors, either autologous or allogeneic, has largely replaced the need for bone marrow stem cell collection. This is discussed in detail in Chap. 55, but suffice it to say that improvements in apheresis technology have led to a much more streamlined, efficient, and comfortable process of collecting stem cells for marrow reconstitution.

Plasmapheresis/Plasma Exchange

Hyperviscosity Syndrome

The clinical syndrome of blood hyperviscosity is observed when there is an excessive amount of circulating immunoglobulin, produced by a clonal plasma cell disorder, most commonly Waldenström's macroglobulinemia (WM) [79, 80]. In WM, there is production of an IgM paraprotein, which is a pentamer and thus very large, and by virtue of its size and intravascular localization, high levels lead to increased serum viscosity [79–81]. Similarly, in IgA myeloma, in which the paraprotein is a dimer, and in myeloma-producing IgG subtype 3, a subtype that can polymerize, high levels can lead to increased blood viscosity [79, 80]. These larger paraprotein molecules remain in the vasculature and do not distribute into the tissues, and thus impact directly on the intravascular blood viscosity [81–83]. Thus, plasmapheresis is highly effective in removing intravascular paraproteins, and a single procedure can result in a significant reduction in the paraprotein level and a significant reduction in serum viscosity [81–84]. This can lead to very rapid resolution of symptoms, and is the initial treatment of choice for patients with symptomatic hyperviscosity. Complete resolution may require 2–3 sessions with each involving a complete plasma exchange.

Symptoms of hyperviscosity may include headaches, blurred vision, retinal hemorrhages, epistaxis, and impaired mentation [84, 85]. Some of the ophthalmologic findings have the appearance of central retinal vein occlusion, and in one study, nine symptomatic WM patients were evaluated by temporal retinal vein blood flow using laser Doppler, before and after plasmapheresis [86]. In this study, plasmapheresis resulted in a significant reduction in serum IgM concentration and serum viscosity (46.5% and 44.7% reductions, respectively) and the appearance of the retinopathy also improved immediately after plasmapheresis. After treatment, the venous diameter decreased in each patient by an average of 15%, and this was accompanied by an average of 55% increase in retinal venous blood speed [86].

It is important to keep in mind that in more recent treatment approaches for WM, with the usage of rituximab as part of the therapy, there is described a phenomenon of rituximab-mediated IgM flare. In patients with already high IgM levels, this can precipitate symptomatic hyperviscosity or other IgM-related complications, such as cryoglobulinemia [84, 87, 88]. Some recommend plasmapheresis for patients with high levels of IgM in anticipation of starting rituximab treatment [84, 88].

Another indication for plasmapheresis is in the management of paraproteinemic neuropathy, which may occur in the context of myeloma, cryoglobulinemia, WM, or monoclonal gammopathy of undetermined significance [5, 89]. This remains a difficult cluster of symptoms and often requires additional treatment of the underlying hematologic disorder.

Cryoglobulinemia

Cryoglobulinemia is a syndrome occasionally observed in hematologic malignancies, and of these, most commonly WM with its clonal IgM protein. Mixed cryoglobulinemia is most commonly associated with hepatitis B and C infections [5]. In this syndrome, the paraprotein or mixed globulins (but including IgM antibodies) precipitate in cooler vascular beds, such as extremities and earlobes, causing damage due to poor perfusion [90]. Again, since the antibodies are largely intravascular, they are amenable to removal by plasmapheresis. Plasmapheresis is efficient in removing these antibodies and is used in all forms of cryoglobulinemia, usually in conjunction with treatment of the underlying illness [5, 84, 91].

The clinical findings are related to poor perfusion due to this precipitation, and may include leg cramps, acrocyanosis, Raynaud phenomenon, ulcers, and purpura. This poor tissue perfusion may lead to infarction or ischemia. Plasmapheresis has been documented to enhance tissue healing after removal of cryoglobulins [92]. One recent analysis of the current literature of plasma exchange in cryoglobulinemia remains critical of this approach, but confirms that the published studies that they reviewed do support the use of plasma exchange, primarily for the bulk removal of the abnormal proteins [93].

ABO Incompatible Stem Cell Transplantation

As discussed in Chap. 56, in the supportive care of transplant patients, transplantation of ABO incompatible grafts can lead to acute and delayed hemolysis and delays, some of which can be quite prolonged, in red cell engraftment. Whereas there are fewer red blood cells in a peripheral blood stem cell collection, there are also more lymphocytes, which may increase the frequency of delayed antibody production by the graft [94]. Apheresis is used to deplete high donor titers of iso-agglutinins, and red cell depletion of the graft can also be accomplished through blood bank reduction techniques. Other approaches are discussed in detail in Chap. 59.

Summary

Apheresis techniques have long been utilized in the management of hematologic malignancies and continue to fulfill a role. Much of the utilization has become specialized to acute situations, when rapid reduction of an abnormal blood component is required for clinical safety and management. The hematologic disease-specific guidelines for utilization presented here are reflective of the hematologic literature and of recent guideline publications. Both the American Society for Apheresis and the British Transfusion Society/British

Society of Haematology have reported guidance for apheresis across the specialties of hematology, nephrology, and neurology [95, 96]. Apheresis remains an important tool in the acute management of some patients with hematologic malignancies and has well-documented benefit in these situations.

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Red Cell Transfusions in Patients with Hematologic Malignancies

53

Karen E. King and Paul M. Ness

Introduction

Although the transfusion of red cells is a relatively common clinical event, patients with hematologic malignancies have special requirements for their red cell products and are at increased risk for certain adverse effects of transfusion. This chapter will provide an overview of many of the basic principles of transfusion medicine with a focus on the issues which are relevant to the restoration of oxygen carrying capacity in the patient with a hematologic malignancy.

Red Blood Cell Components

Currently, the clinical practices of transfusion medicine are based on component therapy, in which a unit of whole blood can be separated into red blood cells, platelets, and plasma. Component therapy is advantageous since it provides maximal use of a limited resource, blood. In addition, with the awareness of transfusion-transmitted diseases and other adverse effects of transfusion, the use of component therapy should limit donor exposures and patients should receive only the blood products which they require. Several types of red cell components are available; they are described next and summarized in Table 53.1.

Table 53.1 Red blood cell components and their indications

Red cell component	Characteristics	Clinical indication
Whole blood	High volume	Replacement of combined red cell and volume deficit (massive blood loss)
	Good flow	
Red blood cells	Lower volume	Replacement of red cell deficit
	Higher hematocrit (65–80%) Good flow with AS-1	
Leukocyte reduced red blood cells	Less than 5×10^6 white cells per unit	Prevention of febrile, nonhemolytic transfusion reactions
		Prevention of alloimmunization
		Prevention of CMV transmission
Washed red blood cells	Plasma depleted	Prevention of severe, allergic transfusion reactions
Frozen red blood cells	Storage for at least 10 years	Storage of rare donor units
	Plasma depleted	

Whole Blood

Due to the predominant use of component therapy and the difficulty in predicting the need for whole blood, most blood banks do not maintain an inventory of whole blood. Obviously, for use in the setting of massive blood loss, a large amount of whole blood would be optimal. Since this inventory is not usually available, red cells in combination with a volume expander should be used. If the volume loss is moderate in amount, a crystalloid, such as normal saline, can be used. Crystalloids have the advantages of being sterile and inexpensive. For more extensive volume replacement, colloids such as albumin [1, 2] and starch solutions are available. These preparations are much more expensive than crystalloids; however, they are preferable over plasma in that they carry no risk of disease transmission. After receiving the equivalent of approximately one blood volume, the

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patient is at risk for a dilutional coagulopathy. At this point, plasma should be administered to replace coagulation factors and platelets should be given [3]. It should be remembered that platelets are suspended in plasma; when giving both platelets and plasma, one can give less plasma taking into account the plasma contained in the platelet product. With the use of platelet additive solutions, these products will contain less plasma than routine platelets.

A unit of whole blood has a volume of approximately 510 mL, consisting of 450 mL of whole blood and 63 mL of an anticoagulant–preservative solution. When this product is collected in CPDA-1, it has a shelf life of 35 days. During the first 24 h of storage, the platelets become nonfunctional [4]. In addition, there is a gradual decrease in the activity of coagulation Factors V and VIII. By day 21 of storage, Factor V levels range from approximately 20 to 100%. At this same time, Factor VIII levels range from about 15 to 50% [5]. The other coagulation factors are stable in stored whole blood.

Whole blood simultaneously replaces both red cells and plasma. Thus it can provide improved oxygen carrying capacity, colloid osmotic pressure, volume replacement, and many of the plasma coagulation factors. Whole blood is appropriately used for massive blood loss such as in trauma or in surgery with extensive blood loss. Although whole blood is not likely to be of major use for patients with hematologic malignancies, there is increased interest among surgeons and treaters of massive hemorrhage for the use of whole blood in massive transfusion protocols; although the platelets in whole blood may have shorter survival than platelets stored at room temperature, whole blood may be more effective in providing rapid hemostatic improvement.

Red Blood Cells

Red blood cells are commonly prepared from a unit of donor whole blood following the removal of most of the plasma [6], which may be achieved by either sedimentation or centrifugation. The removed platelet-rich plasma may then be used for the preparation of another component, such as fresh frozen plasma or platelets. Red blood cells can also be produced by apheresis techniques, in which up to 2 units can be harvested from one donor at a single donation [7]. A unit of red cells has an average volume of 300 mL and has a hematocrit ranging between 65 and 80%. An additive solution, such as AS-1 or AS-3, is often used to extend the storage life to 42 days. The additive solution will decrease the hematocrit, so that the product will have flow properties similar to whole blood [8].

Red blood cells are indicated for the improvement of oxygen carrying capacity especially if it is an isolated problem. With their relatively high hematocrit, red blood cell units can efficiently provide red cells in a small volume. They are the product of choice in the setting of chronic anemia from bone marrow failure. Red blood cells are especially helpful in patients with cardiovascular compromise who may not tolerate the additional volume of whole blood.

Leukocyte Reduced Red Blood Cells

An average unit of red blood cells contains approximately 2×10^9 to 5×10^9 leukocytes [9]. Several methods have historically been used to reduce the leukocyte content of a unit of red cells; these methods have varied in their capacity for leukocyte reduction. All leukocyte reduced red cell products must retain at least 85% of their original red cell content and their total white cell content must be less than 5×10^6 [6].

Advanced leukocyte removing filters have been developed to reduce the leukocyte content of red cells. These “third generation” filters use two mechanisms: a barrier on the basis of pore size and retention by adsorption; thus, they are sometimes called leukocyte adsorption filters [10]. Several studies have evaluated the efficacy of these filters and have found that they can reliably reduce the white cell content of a unit of red cells by as much as 99.9%, which is a 3 log reduction [11–15]. Prestorage leukocyte reduction has the additional advantage of preventing cytokine accumulation. Both whole blood derived red cells and red cells harvested by apheresis can be leukocyte reduced.

There are several indications for the use of leukocyte reduced red cells, including the prevention of febrile, nonhemolytic transfusion reactions, the prevention of alloimmunization to HLA antigens (especially for patients who will require future platelet transfusions or a possible progenitor cell transplant), and the prevention of cytomegalovirus (CMV) transmission. In addition, there is evidence that transfusion-associated immunomodulation is mediated by contaminating white cells in blood products and these potential complications would be prevented with the use of leukocyte reduced blood products [16]. Many hospitals have moved to a universal leukocyte reduction policy, although some hospitals only provide leukocyte reduced blood for patients meeting specific indications. We believe most patients with hematologic malignancies will benefit from leukocyte reduced red cells.

Washed Red Blood Cells

Washed red blood cells are prepared by either manual or automated washing with isotonic saline. Washing red cells removes plasma and some of the leukocytes and platelets. This procedure can be repeated several times depending on the indication and the degree of plasma removal necessary, but there is red cell loss with each washing. Since the washing

procedure is performed in an open system, washed red cells must be transfused within 24 h of initiation of manipulation.

Since washing primarily removes the plasma proteins from red cells, the main indication for washed red cells is the prevention of severe allergic transfusion reactions. These severe allergic transfusion reactions are felt to be due to recipient antibodies directed against donor plasma proteins. Washing is indicated when a patient has recurrent severe allergic reactions that are refractory to antihistamine treatment. Patients with IgA deficiency and antibodies to IgA can have anaphylactic reactions to plasma containing IgA [17]. These patients may tolerate red cells which have undergone several washes [18] or they may require products from IgA deficient donors [19]. It has been suggested that patients with paroxysmal nocturnal hemoglobinuria (PNH) receive washed red cells. Since the red cells in PNH are more sensitive to complement, it was hypothesized that washing removes the complement and the washed red cells would be less likely to exacerbate the hemolytic process of PNH [20]. One retrospective study has shown that patients with PNH do not require washed red cells [21, 22].

Frozen Red Blood Cells

Red blood cells may be frozen using glycerol as a cryopreservative. The concentration of glycerol used in the freezing procedure depends on the rate and the temperature of freezing [23, 24]. Other contaminating cellular elements are destroyed in the freezing process, except for a small number of lymphocytes [25]. Thus, frozen red cells contain a decreased number of leukocytes. When the cells are needed for transfusion, the unit is thawed and washed repeatedly with progressively less hypertonic saline solutions to remove the glycerol from the cells. If the process is not performed appropriately, hemolysis will occur upon transfusion. The repeated washings remove associated plasma and any other debris. The red cells are resuspended in isotonic saline with glucose. Once thawed, these red cells have been shown to be more fragile than conventional liquid-stored red cells [26]. Frozen red cells may be stored for at least 10 years; however, they have been shown to have good viability even after longer periods of storage. Once thawed, the product is an open system and will outdate in 24 h.

This process is expensive and the indications for this product are limited. The major indication for frozen red cells is for preservation of rare donor units required by patients with unusual phenotypes who have developed multiple alloantibodies. These patients should be encouraged to donate autologously when they are clinically able and have their cells frozen for future needs. Autologous blood may also be frozen when a surgery date is postponed or if more blood is needed than can be donated within the time limitations of liquid storage (35–42 days). Frozen red cells have been used in patients with aplastic anemia who are awaiting bone marrow transplantation, since frozen cells have shown the least adverse effect on subsequent graft survival [27]. Leukocyte reduced red cells have replaced frozen red cells for this indication. Occasionally, there are patients with severe, repeated febrile nonhemolytic transfusion reactions who fail medications and other forms of leukocyte reduction, and these patients may have uneventful transfusions when given frozen red cells.

Modifications and Attributes of Red Blood Cell Products

Red cells can have additional modifications or attributes which meet the special needs of patients with hematologic malignancies.

Gamma Irradiation

Gamma irradiation is used to prevent transfusion-associated graft-versus-host disease. This disease occurs when immunocompetent lymphocytes are transfused which are capable of engrafting and reacting against the host tissues [28]. This entity is of concern in any patient who is severely immunocompromised. Transfusion-associated graft-versus-host disease is also a risk in situations of partial HLA identity between the donor and the recipient [29, 30]. Because transfusion-associated graft-versus-host disease is associated with significant mortality, prevention is critical. Thus, gamma irradiation is recommended for all cellular products that are given to patients at risk for transfusion-associated graft-versus-host disease, including those with congenital immunodeficiency syndromes, recipients of units from relatives, recipients of HLA selected products (either by typing or crossmatching of platelets), and bone marrow or progenitor cell recipients. It has been shown that patients with chronic lymphocytic leukemia (CLL) who are undergoing treatment with fludarabine are at risk for transfusion-associated graft-versus-host disease [31, 32]. Additionally, other patients receiving fludarabine therapy, such as those with systemic lupus erythematosus, are also at risk for transfusion-associated graft-versus-host disease [33]. Some oncology centers routinely gamma irradiate all cellular blood components to avoid the often fatal complications of transfusion-associated graft-versus-host disease.

Gamma irradiation inactivates immunocompetent lymphocytes, preventing proliferation of transfused T lymphocytes. It is recommended that a minimum of 25 Gy be delivered to the center of the irradiation field with at least 15 Gy delivered to all parts of the blood

component [6, 34]. Although this dosage should not affect the function of the red cells, irradiation damages red cell membranes and does have an effect on the overall viability, requiring a shortened outdate. Due to the devastating repercussions of this disease, the lack of effective treatment, and the relative ease of irradiation, prevention is the focus and most centers err on the side of irradiating for less than well-documented indications. Gamma irradiation does not produce a leukocyte reduced product or a CMV-safe product.

Cytomegalovirus Seronegative Products

Cellular blood products can be tested for antibodies against CMV by a variety of methods. Products that are seronegative for CMV are recommended in situations in which immunosuppressed patients would be at risk for getting primary CMV infection. Patients at risk for transfusion-transmitted CMV include CMV seronegative patients undergoing bone marrow transplantation from a CMV seronegative donor and CMV seronegative patients with AIDS [35, 36].

Since this virus is associated with leukocytes, it has been proposed that leukocyte reduced blood products would be useful in the prevention of CMV transmission. Studies have shown that leukocyte reduction by filtration is helpful in preventing CMV transmission [37–39]. One study has shown that filtration can remove any detectable CMV DNA from previously positive red cell products even following amplification by the polymerase chain reaction (PCR) technique [40]. A randomized study comparing seronegative blood products and leukocyte reduced products using bedside filters has shown that these products are equivalent in preventing CMV infection in patients undergoing marrow transplantation [41].

Controversy exists in both the literature and in clinical practice regarding the role of leukocyte reduced blood products in clinical situations requiring CMV-safe blood [42, 43]. Currently, many institutions are using leukocyte reduced blood products when CMV seronegative products are not available. Other institutions are routinely using leukocyte reduced and CMV seronegative products as equally CMV safe. When leukocyte reduced red cell products are used to prevent CMV transmission, the red cells must have less than 5×10^6 leukocytes.

Special Clinical Situations for Red Cell Transfusion

Although most of the principles of transfusion medicine are applicable to patients with hematologic malignancies, there are a few unique situations which should be noted.

Indications for Red Cell Transfusion

Due to the risks of transfusion, the indications for transfusion should be clear and unnecessary transfusion should be avoided [44]. Frequent reference is made to a transfusion trigger or a suggested minimum hemoglobin or hematocrit level at which red cells are transfused. Obviously, the need for red cells or increased oxygen carrying capacity depends upon numerous factors which are specific to the individual patient [45]. The etiology of the low hemoglobin is crucial in this evaluation. Patients with chronic anemias which have been well compensated will tolerate a more severe degree of anemia than the patient who has suffered an acute blood loss. The patient's underlying health condition is also important in deciding when to transfuse. A young and relatively healthy person with good cardiovascular function will tolerate a lower hemoglobin than an older patient with impaired cardiac function [46, 47].

Previously, it was felt that perioperative patients should have a hemoglobin of at least 10 g/dL. A National Institutes of Health Consensus Development Conference on perioperative red blood cell usage has recommended that the transfusion trigger be changed to a hemoglobin of 7 g/dL [48]. Currently, there is no single transfusion trigger that applies to all patients. In patients with hematologic malignancies, the hematocrit level should be maintained to alleviate the symptoms of anemia and will vary with the patient's age and other medical problems. A landmark study by Carson et al. in hip fracture patients demonstrated no advantages in providing more red cells by liberal triggers of 8–9 g/dL than conservative triggers of 9–10 g/dL [49]. Similar studies have now been performed in many other medical and surgical settings with similar results showing no advantage to liberal red cell transfusion practices [50]. A pilot study in patients with acute leukemia also showed no advantage to liberal red cell transfusion practices, and hopefully other studies will be performed to confirm these results in patients with other hematologic malignancies [51]. Although most studies have focused on other patient populations, it is reasonable to extrapolate that patients with hematologic malignancies will tolerate hemoglobin levels between 7 and 10 g/dL [52]. More liberal, higher transfusion thresholds may be important in patients with underlying cardiac disease [53]. A pilot study in patients with cardiac ischemia and anemia suggested that patients had better outcomes with conservative triggers of 9–10 g/dL [54] and a larger adequately powered study is now underway to answer this critical question.

The measured response to a transfusion varies depending upon the patient. It is generally accepted that transfusion of 1 unit of red cells should increase the hematocrit by 3% or the hemoglobin by 1 g/dL. The FDA requires 75% of the transfused red cells in the circulation at 24 h, when evaluating collection systems and anticoagulant–preservative solutions.

Hyperviscosity

Hyperviscosity becomes problematic in two clinical situations involving patients with hematologic malignancies. Patients with multiple myeloma and plasma cell dyscrasias can have difficulties due to hyperviscosity. These patients are frequently anemic and it is difficult to determine if their symptoms of poor oxygenation are due to hyperviscosity or their severe anemia. A relative serum viscosity measurement should be determined; many patients will become symptomatic at a relative serum viscosity above 4. A patient with hyperviscosity will not tolerate red cell transfusions unless their viscosity is first reduced. In this situation, plasmapheresis can be performed to reduce the amount of circulating paraprotein which is creating the hyperviscosity [55, 56].

Hyperleukocytic leukostasis occurs in patients with markedly elevated white cell counts, frequently due to acute myelogenous leukemia. Although white cells do not usually contribute significantly to whole blood viscosity, hyperleukocytosis with malignant white cells can have associated hyperviscosity since the malignant white cells may not have normal flow characteristics. These patients frequently present with hyperleukocytosis, anemia, and evidence of poor perfusion which may be due to leukostasis or to infiltration of blood vessels by leukemic cells. In these patients, the white cell count should be reduced by leukocytapheresis before the patient is given red cell transfusions [52]. If leukocytapheresis technology is not available, the patient's white cell count can be reduced by manual whole blood exchange or chemotherapy [57, 58].

Bone Marrow and Progenitor Cell Transplants

Patients who are undergoing bone marrow or progenitor cell transplantation have several special needs. They will be transfusion dependent until engraftment occurs. All bone marrow transplant patients should receive irradiated red cells to prevent the development of transfusion-associated graft-versus-host disease. In addition, CMV negative patients who receive autologous bone marrow transplants, autologous peripheral stem cells, or CMV negative allogeneic transplants should receive CMV-safe blood products.

In cases of bone marrow transplantation in which there is a major ABO incompatibilty (a group O patient given bone marrow from a group A, group B, or group AB donor), the marrow infusion can be depleted of contaminating red cells. When engraftment occurs, the transplanted marrow will begin to produce red cells with A and/or B substance and the recipient may still be producing ABO antibodies. In this situation, the new red cells may be hemolyzed by the ABO antibodies. This period of hemolysis may be brief or it may last for an extended period of time. If transfusions are required during this period, the patient should be given group O red cells. Of course, these cells should be irradiated and CMV seronegative cells may also be indicated.

Minor ABO incompatibilty in bone marrow transplants occurs when group O donor marrow is transplanted into a non-group O recipient. Plasma should be removed from the marrow product to reduce the amount of incompatible ABO antibodies given to the recipient. However, as in the case of solid organ transplants, passenger lymphocyte syndrome may occur and contaminating lymphocytes are capable of producing anti-A and anti-B. In many of these cases, hemolysis may occur [59]. If transfusions are required, irradiated group O red cells should be given.

Autoimmune Hemolytic Anemia

Autoantibodies directed against red cells occur frequently in patients with hematologic malignancies. Patients with CLL are notorious for the development of red cell autoantibodies, often with an autoimmune hemolytic anemia. It has been estimated that 10–20% of patients with CLL will develop red cell autoantibodies at some time during the course of their disease. The mechanism of autoimmune hemolytic anemia in patients with CLL is controversial. Some believe that these patients have an imbalance in their T helper and T suppressor lymphocyte subsets. One study showed that the neoplastic clones of CLL were capable of in vitro production of monoclonal antibodies which could bind red cells. This study suggests that the autoantibodies may actually be produced by the malignant clonal B cell population [60]. The use of fludarabine in the treatment of CLL has been associated with the development of severe autoimmune hemolytic anemia. The mechanism of this association is unclear, but it has been proposed that fludarabine results in a further disruption of the T lymphocyte subsets [61, 62].

Red cell autoantibodies are detected in the laboratory by the direct antiglobulin test (DAT, direct Coombs test). This test can detect the presence of IgG and/or complement on the surface of red cells. The indirect antiglobulin test (IAT, indirect Coombs test) may reveal a panagglutinin in the serum of patients with an autoantibody. Warm autoimmune hemolytic anemia is usually due to IgG antibodies which react at 37 °C and may or may not fix complement. Thus, in warm autoimmune hemolytic anemia, the DAT will reveal IgG and/or complement on the red cells. Cold autoantibodies (cold agglutinin syndrome) are usually IgM antibodies which react at 4 °C and are able to fix complement. In cold agglutinin syndrome, the DAT will show the presence of complement on the red cell surface with an absence of IgG.

Clinically, it is important to determine if a patient with a hematologic malignancy and red cell autoantibodies is

actually hemolyzing. The diagnosis of autoimmune hemolytic anemia requires both the laboratory features and the clinical evidence of hemolysis. Patients with autoimmune hemolytic anemia may require red cell transfusions. It is important to rule out the presence of underlying red cell alloantibodies which may be masked by the panagglutinin, since alloantibodies may complicate transfusion therapy. Even after underlying alloantibodies have been excluded, the panagglutinin will result in incompatible crossmatches. These patients should be transfused with red cells which lack any alloantigens for which the patient has corresponding underlying alloantibodies or phenotypically matched red cells [63]. Use of leukocyte reduced red cells is recommended to minimize the occurrence of febrile nonhemolytic transfusion reactions which would confound an already difficult clinical situation. We recommend that these patients be transfused when they clinically require red cells, with the understanding that the transfused cells will have a shortened survival comparable to the patient's own red cells.

Erythropoiesis Stimulating Agents

Erythropoietin is a hormone which is capable of promoting the proliferation and maintaining the viability of erythroid progenitor cells. This hormone is produced predominantly by renal peritubular interstitial cells and also by the liver [64–66]. In 1985, the erythropoietin gene was cloned and expressed [67, 68]. Erythropoiesis stimulating agents, including recombinant erythropoietin and the closely related recombinant darbepoetin, have been used in the treatment of anemias secondary to malignancies, including multiple myeloma and myelodysplastic syndromes [69, 70]. In responsive patients, erythropoiesis stimulating agents can effectively alleviate anemia and reduce transfusion requirements [71, 72]. Due to concerns about erythropoiesis stimulating agent therapy being associated with tumor progression, venous thromboembolism, and/or survival, an update committee of the American Society of Hematology/American Society of Clinical Oncology has reviewed the literature related to erythropoiesis stimulating agent use in adult patients with cancer [72]. Their revised recommendations include weighing the risks and benefits of erythropoiesis stimulating agents with the risks and benefits of transfusion for the specific patient undergoing myelosuppressive chemotherapy who has a hemoglobin <10 g/dL. If used, erythropoiesis stimulating agents should be used at the lowest dose which successfully increases the patient's hemoglobin to a level where transfusion is no longer required. For patients who do not respond to therapy, the erythropoiesis stimulating agent should be discontinued after 6–8 weeks.

Red Blood Cell Preservation and Storage

Whole blood and red cell components are stored in a liquid state at a temperature between 1 and 6 °C. Red cells are stored in an anticoagulant–preservative solution with or without an additive solution. Most anticoagulant–preservative solutions contain trisodium citrate, citric acid, dextrose, and phosphate (ACD, CPD, and CP2D). The appropriate storage time or shelf life for red cells is based on the FDA criteria that 75% of the transfused cells should be in circulation 24 h after the transfusion. Red cells stored in a standard anticoagulant–preservative solution, such as ACD, CPD, and CP2D, have a shelf life of 21 days. CPDA-1 contains adenine and blood collected in CPDA-1 has a shelf life of 35 days. Additive solutions, such as AS-1 or AS-3, contain additional dextrose, adenine, and sodium chloride. AS-1 also includes mannitol. Red cells stored with an additive solution may be stored for up to 42 days [6].

Adenosine Triphosphate

It has been shown that adenosine triphosphate (ATP) levels in stored red cells correlate with the viability of the red cells following transfusion. As the red cell ATP level drops during storage, the red cells undergo changes in shape from disc to sphere followed by an increase in cellular rigidity. There is also a loss of cell membrane with decreased volume [73]. Dextrose in the preservative solution is crucial in supporting the glycolytic pathway for continued generation of ATP. The additional adenine in CPDA-1 and in the additive solutions provides substrate for the formation of ATP. Although other factors may also influence the overall viability of red cells following storage, ATP remains the best defined factor as an indicator of acceptable red cell survival without performing red cell survival studies.

2,3-Diphosphoglycerate

2,3-Diphosphoglycerate (2,3-DPG) plays a crucial role in the delivery of oxygen from the red cell by influencing the position of the oxygen dissociation curve. With high 2,3-DPG levels, more oxygen is released at the same PO2. With lower red cell 2,3-DPG levels, there is greater affinity of hemoglobin for oxygen and less oxygen is delivered to the tissues. Although 2,3-DPG levels decrease in stored red cells, there is restoration of these levels in vivo following transfusion. In fact, studies have shown that over 50% of 2,3-DPG is restored within the first 24 h following transfusion. Based on these observations, blood at any point in its permissible storage period is acceptable for patients with hematologic malignancies [74].

Byproducts of Red Cell Storage

Due to the presence of citrate in the anticoagulant–preservative solutions, the possibility of citrate toxicity must be considered in situations of massive transfusion. This clinical entity rarely occurs, but the risk is greater in instances of impaired liver function, since the liver metabolizes citrate. The danger of infusing high volumes of citrate over a short period of time is due to the chelation of calcium by citrate. Consequently, the patient's ionized calcium level may drop and lead to cardiac arrhythmias. Usually, this adverse effect can be treated by slowing the infusion rate or by the administration of calcium gluconate. If citrate toxicity occurs and calcium supplementation is required, it is recommended to follow ionized calcium levels, since hypercalcemia can also lead to cardiovascular sequelae [75].

With prolonged storage of blood, the level of extracellular potassium gradually increases. Since the sodium–potassium pump is relatively nonfunctional at red cell storage temperatures, the intracellular and extracellular potassium equilibrate resulting in an increase in the extracellular potassium. This situation is exacerbated by any hemolysis which occurs during the storage period. The plasma potassium concentration of red cells stored in CPDA-1 is approximately 78 mEq/L on day 35 as compared to 5 mEq/L in freshly collected blood [76]. Of course, the total plasma volume of a unit of red cells is about 70 mL, yielding a total potassium load of only 5.5 mEq in each unit of red cells. The vast majority of patients are able to tolerate this increase in potassium; however, in some situations, this elevation in potassium may cause cardiac complications. In patients with renal failure and hyperkalemia or in patients with very low blood volumes, the increased potassium may be a problem. In this situation, fresher units should be selected, or if not available, an older unit of red cells may be washed.

During the storage process, microaggregates of platelets, white cells, and fibrin can form. These microaggregates may measure up to 200 μm in diameter [77]. The clinical impact of these microaggregates remains controversial. Some studies have shown that in massive transfusion, the degree of hypoxia is related to the amount of blood transfused, thus implicating microaggregates as the cause of the pulmonary dysfunction [78, 79]. Others have argued that in cases of traumatic shock, massive transfusion, and pulmonary dysfunction, microaggregates may be contributory to the hypoxia, but their role is relatively minor [80]. Animal studies have been unable to confirm the role of microaggregates in this clinical situation [81]. Some have suggested the use of microaggregate filters when more than 5 units have been transfused [82], but one should be aware that these filters slow the rate of infusion.

The 1960s brought about the development of the plastic blood bag for the storage of red cells. This was a major innovation and a significant step toward the current trend of component therapy. These plastic bags are made from polyvinylchloride containing the plasticizer di(2-ethyl)phthalate (DEHP). DEHP is lipophilic and is known to leach out of the bag during storage [83, 84]. Since the development of this bag, there has been much concern about its safety. Numerous animal studies have shown significant toxicity ranging from a shock lung-like picture to teratogenicity [85]. The AABB issued a statement in June 2003 acknowledging the risks of the plasticizer, DEHP, in polyvinylchloride blood bags, and recognizing the need for development and availability of newer plasticizers for blood bags [86]. Other potential materials have been proposed for use in blood storage bags, including polyvinylchloride plasticized with butyryl-*n*-trihexyl-citrate (BTHC). The BTHC undergoes less leaching and the bags have good red cell recovery [87, 88].

Adverse Effects of Red Cell Transfusions

There has been an ongoing increased awareness of the potential adverse effects of blood transfusions. Although this heightened awareness was initially stimulated in large part by transfusion-transmitted human immunodeficiency virus (HIV), there are several other potential deleterious effects of transfusion which can have devastating implications. These adverse effects are described next and are shown in Table 53.2.

Table 53.2 Adverse effects of red cell transfusions

Adverse effect
Transfusion-associated circulatory overload
Iron overload
Transfusion reactions
Hemolytic transfusion reactions
Septic transfusion reactions
Febrile nonhemolytic transfusion reactions
Allergic transfusion reactions
Transfusion-related acute lung injury
Transfusion-transmitted diseases
Hepatitis B
Hepatitis C
Cytomegalovirus
Human immunodeficiency virus
Other microbiologic agents
Transfusion-associated graft-versus-host disease
Alloimmunization
Antibodies to red cell antigens
Antibodies to neutrophil antigens
Antibodies to HLA antigens
Immunomodulation

Transfusion-Associated Circulatory Overload

Previously referred to as volume overload, transfusion-associated circulatory overload is a problem in patients with cardiovascular compromise. This complication can result in congestive heart failure and pulmonary edema. Patients should be followed closely and given diuretics if this problem arises. The red cells should be infused at a slow rate or in smaller volumes.

Iron Overload

Iron overload can occur in chronically transfused patients. Each unit of red cells contains 200–250 mg of iron. Following approximately 100 units of red cells, the problem of iron accumulation may occur. Iron will accumulate within the mitochondria which ultimately may affect function in the heart, liver, and endocrine glands. Patients with hematologic malignancies who have had extensive red cell transfusion support are at risk for iron overload. Iron chelation therapy should be considered to reduce their iron burden and potentially improve outcome and survival [89, 90]. Currently, deferoxamine and deferasirox are approved for use in the USA.

Transfusion Reactions

Acute hemolytic transfusion reactions are the most severe transfusion reactions and they have potentially devastating outcomes. These reactions are mediated by antigen–antibody interaction. The most severe hemolytic transfusion reactions occur when ABO incompatible blood is transfused. Clinically, patients develop fever, chills, nausea, and back pain. Patients have hemoglobinuria, hemoglobinemia, and hypotension progressing to shock. The process leads to complement activation, initiation of the coagulation system, and release of bradykinin, catecholamines, and vasoactive amines. Disseminated intravascular coagulation develops; in surgical patients, the coagulopathy may be the first indication of the transfusion reaction. Treatment of these reactions includes supportive care, blood pressure support, fluids and diuretics to maintain renal perfusion, and dialysis if acute renal failure develops.

Septic transfusion reactions are usually related to a platelet product and not a red cell component. Since platelets are stored at room temperature for up to 5 days, they are particularly susceptible to bacterial contamination. When red cells are involved in a septic transfusion reaction, the organisms are usually Yersinia enterocolitica or the psychrophilic pseudomonads. Yersinia enterocolitica is a gram-negative bacillus that clinically causes enterocolitis. The growth of the organism is enhanced by the presence of iron and it grows well at 4 °C. The organism rapidly multiplies after 21 days of storage, potentially giving older units very high bacterial concentrations. Thus, red cells provide an ideal environment for this organism. *Pseudomonas putida* and *Pseudomonas fluorescens* have also been implicated in septic transfusion reactions. These organisms are gram-negative rods and they grow well at 4 °C [91, 92].

Febrile nonhemolytic transfusion reactions clinically occur when a patient experiences a rise in temperature of 1 °C or more reaching an oral temperature of greater than or equal to 38 °C or chills and rigors in association with a transfusion and without any other explanation. These reactions are generally felt to be due to leukoagglutinins or recipient antibody against donor leukocytes [93–95]. Patients with recurrent febrile, nonhemolytic transfusion reactions are given leukocyte reduced blood products [96, 97]. In many cases, leukocyte reduced blood products are effective in preventing further febrile transfusion reactions [98]. Some patients, however, continue to have reactions despite leukoreduction; in fact, one study has shown that in patients receiving platelet transfusions, leukoreduction had limited efficacy in the prevention of febrile reactions [99]. There has been much interest in the role of cytokines in the pathogenesis of febrile transfusion reactions which cannot be attributed to leukoagglutinins [100]. Many reactions to platelets have now been shown to be due to the cytokines remaining in the plasma derived from contaminating leukocytes in the product [101]. Prestorage leukocyte reduction can prevent the production and accumulation of cytokines in stored products.

Mild allergic transfusion reactions or urticarial reactions present with cutaneous symptoms of itching and hives. These reactions are often treated with antihistamines, but with limited efficacy. If the reactions become more severe in nature, washed blood products may be helpful in further prevention. These reactions are felt to be due to antibodies in the recipient against donor plasma proteins.

Severe allergic reactions or anaphylactic reactions present clinically with bronchospasm, respiratory distress, and vascular instability leading to shock and loss of consciousness. Treatment includes supportive care and epinephrine. Steroid therapy may be useful. These reactions are often felt to be related to recipient antibodies against donor plasma proteins. These reactions also occur in patients who are IgA deficient and have antibodies to IgA. It is recommended that patients with IgA deficiency and antibodies to IgA receive blood products from IgA deficient donors or washed red cells.

Transfusion-related acute lung injury (TRALI) occurs in patients who develop new onset of acute lung injury with hypoxemia during or within 6 h of a transfusion. Clinically, patients present with respiratory distress, hypoxia, hypotension, fever, and bilateral pulmonary edema [102]. Patients should have no evidence of left atrial hypertension

and circulatory overload or any other temporally associated alternative risk factor for acute lung injury. It is felt that these reactions are mediated by the infusion of HLA- or granulocyte-specific antibodies present in the transfused product which reacts with the recipient's antigens [103]. Because these antibodies are associated with multiparous females, the exclusion of female plasma donors is becoming more prevalent [104]. An alternate pathogenesis involves the oxidation of membrane lipids by donor leukocytes which may prime the recipient neutrophils, such that a second stimulus can cause the release of vasoactive mediators [105]. Treatment consists of supportive care, including blood pressure and respiratory support.

Transfusion-Transmitted Diseases

As the number of documented transfusion-transmitted diseases has grown, donor testing has expanded in an effort to preemptively identify these pathogens and prevent transfusion [106]. Despite the routine testing of all blood products for hepatitis B surface antigen (HBsAg), antibodies to hepatitis B core (anti-HBc), hepatitis B virus nucleic acid amplification testing (HBV NAT), antibodies to hepatitis C virus (anti-HCV), and hepatitis C virus nucleic acid amplification testing (HCV NAT), transfusion-associated hepatitis can still occur. The risk of transfusion-associated hepatitis B was estimated to be 1 in 220,000 components transfused in 2003 [107]. The incidence of transfusion-associated hepatitis C has been greatly reduced with the development of better testing. The estimated risk of hepatitis C virus transmission by screened blood was 1 in 1,800,000 units transfused in 2005 [108]. More recent publications suggest that the risk of HBV and HCV are less than 1 in 1,000,000 [109].

Transfusion-associated AIDS was first reported in late 1982 [110]. Serologic testing for HIV became available in 1985 [111]. Currently, blood is tested for HIV serology, including HIV-1 and HIV-2 antibodies, and by HIV-1 nucleic acid amplication testing. The estimated risk of HIV transmission from screened blood was 1 in 2,300,000 units transfused in 2005 and the risk continues to fall with current practices [108].

CMV is a herpes virus. It is a double stranded, DNA virus, capable of remaining latent in tissues following primary disease. Clinical disease due to this virus may be on the basis of primary infection, reactivation, or reinfection. CMV is felt to be harbored within white cells. Clinical CMV disease can be quite variable; however, primary disease is usually more severe than reactivation. Although there have been significant improvements in pharmacologic approaches to the prevention of CMV, this infection remains a significant source of disease and mortality in recipients of allogeneic progenitor cell transplants [112]. In patients at risk for CMV infection, CMV-safe blood products should be administered. Because of the presence of the virus in white cells, leukoreduction has become an alternative approach to seronegative blood products for some patient populations.

Other infectious risks continue to be of concern for transfusion recipients. Donor blood is now tested for West Nile Virus as a result of a transfusion epidemic that was documented [113]. Babesia transmission has been noted from donors in New England and testing programs are being studied [114]. Dengue virus is a problem in Puerto Rico [115] and donor blood in the US is now tested to prevent transfusion transmission of Zika [116].

Although pathogen inactivation is now available for platelets and plasma in the US, there are no licensed red cell products that have undergone pathogen inactivation processing. Two commercial systems are in development and will undergo clinical trials in the near future [117]. A recent study of one system using riboflavin and UV light was demonstrated to reduce transfusion-transmitted malaria in an endemic area of Africa [118]. In addition to eliminating most of the infectious risks of viruses, bacteria, and parasites, evolving pathogen inactivation systems will also inactivate white cells in blood components, eliminating the need for gamma irradiation and may also reduce alloimmunization to platelets [117].

Transfusion-Associated Graft-Versus-Host Disease

Transfusion-associated graft-versus-host disease is a virtually fatal complication of transfusion. The disease occurs when immunocompetent lymphocytes are given to an immunoincompetent recipient. The disease presents usually 7–10 days after transfusion. Patients develop fever and an erythematous, maculopapular rash. The disease also affects the gastrointestinal tract, liver, and lymphoid tissues, especially the thymus [119]. In patients who have not undergone a bone marrow transplant, the disease can involve the bone marrow and can produce a profound aplasia. This disease has a more aggressive and rapid course than the graft-versus-host disease following bone marrow transplantation. Patients usually die within 3 weeks due to infection or a bleeding complication [28].

Transfusion-associated graft-versus-host disease has been reported in several clinical situations including patients with congenital immunodeficiency syndromes such as severe combined immunodeficiency syndrome (SCIDS). Patients at risk for transfusion-associated graft-versus-host disease include those undergoing bone marrow or progenitor cell transplantation and those receiving products donated by a blood relative who may share one HLA haplotype. All of these patients should receive gamma irradiated cellular blood

products [28]. Additionally, there are several reports of transfusion-associated graft-versus-host disease in patients with CLL being treated with fludarabine [31, 32]. Patients with malignancies are at variable degrees of risk for transfusion-associated graft-versus-host disease, including patients with Hodgkin's disease, non-Hodgkin's lymphomas, leukemia, and some solid tumors. In some institutions, all patients with hematologic–oncologic diseases receive irradiated cellular blood products to eliminate the possibility of this transfusion-related complication. Leukoreduced red cells contain enough remaining white cells to initiate graft-versus-host disease and should not be used instead of gamma irradiation for patients at risk.

Alloimmunization

Since red cell products contain some contaminating white blood cells, patients who receive red cells are at risk for the development of alloantibodies directed against red cell, neutrophil, platelet, and/or HLA antigens. The development of antibodies is multifactorial and involves variables such as the immunogenicity of the antigen, the immune competence of the patient, and the number of exposures.

Retrospective studies have shown that patients with lymphocytic leukemia have a markedly decreased rate of development of red cell alloantibodies. In two studies, none of the 112 total patients became alloimmunized to red cell antigens. It is unclear if this decrease in the incidence of antibody formation is related to the disease or the treatment. Increased rates of alloimmunization to red cell antigens were seen in patients with chronic myeloproliferative diseases (25%) and the myelodysplastic syndromes (31.3%). Intermediate rates of alloimmunization were found in patients with multiple myeloma (11.8%) and the myeloid leukemias (16%) [120–122].

The formation of anti-leukocyte antibodies is of interest because of their role in the pathogenesis of febrile nonhemolytic transfusion reactions. Patients who are multiply transfused may develop complications of repeated febrile nonhemolytic transfusion reactions which can be attributed to anti-leukocyte antibodies; these patients should receive leukocyte reduced red cells. Many of these patients will have uneventful transfusions with leukocyte reduced ($<5 \times 10^6$ white cells) red cells, especially with prestorage leukoreduction [98].

Due to the presence of white cells in red cell products, the multiply transfused patient is at risk for the development of anti-HLA alloantibodies which can complicate platelet transfusion therapy and result in platelet refractoriness. Several studies have shown that multiply transfused patients with hematologic malignancies have reduced HLA alloimmunization and reduced platelet refractoriness when given leukocyte reduced red cell and platelet products [123–126]. A large multicenter clinical trial, the Trial to Reduce Alloimmunization to Platelets (TRAP), compared various strategies to reduce alloimmunization in patients with leukemia. This study confirmed that the use of leukocyte reduced blood products results in decreased alloimmunization to platelets [127]. Further, a Canadian study performed a retrospective analysis of 13,902 platelet transfusions occurring before and after the introduction of universal prestorage leukoreduction of red cell and platelet products [128]. A total of 617 patients undergoing chemotherapy and stem cell transplantation were evaluated. The authors found a reduction in alloimmunization, refractoriness, and the requirement for HLA-matched platelets following the implementation of universal leukoreduction.

Immunomodulation

The effect of transfusion therapy on immune function has generated considerable controversy. Some feel that blood transfusions have an immunomodulatory effect resulting in transfusion-induced immunosuppression. Some studies have shown an increase in tumor recurrence in patients who have been transfused although other studies have shown no effect [129]. The mechanism of transfusion-induced immunosuppression is unclear, although tolerance and anergy have been proposed as possible mechanisms [130]. Animal studies have shown that the tumor growth enhancement associated with transfusions is mediated by donor leukocytes. They also found that the use of leukocyte reduced blood products ameliorates this tumor enhancing effect in animals [131]. The use of leukocyte reduced blood products for leukemic patients has previously been greatly debated, because it has been suggested that the donor leukocytes may exert a graft-versus-leukemia effect. Subsequent studies have failed to show a graft-versus-leukemia effect of transfused leukocytes and others have seen no difference in duration of first remissions in leukemic patients receiving leukocyte reduced blood products [132, 133]. The Finnish Leukemia Group studied patients with acute myeloid leukemia and found that the patients receiving leukocyte reduced blood products had longer median relapse-free survival, shorter periods of aplasia with decreased blood product usage, and decreased occurrence of infections [134]. Despite continued research in this area, definitive evidence of transfusion-associated immunomodulation remains elusive and the controversy continues [135].

Adverse Effects of Prolonged Blood Storage

Red cells stored in anticoagulant–preservative solutions undergo biochemical and physiological changes that are well known but current FDA guidelines still permit 42 days

of red cell storage [136, 137]. An alarming report of cardiac surgery patients suggested that patients receiving older blood had increased mortality and complications ignited considerable interest in this area [138] and led to many studies of blood storage to investigate possible reasons for toxicity [139] and many clinical trials attempting to reproduce the results from the earlier retrospective study [50]. Many clinical trials, including a large study of over 30,000 patients have failed to demonstrate adverse effects on patients receiving blood of any age, and showing no particular advantage to fresher red cells [140]. There is some data suggesting that blood at the end of the storage period may cause some adverse effects; if the in vitro data are confirmed with clinical studies, limiting the storage period of additive red cell to 35 days may become a prudent option [141–144].

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54 Platelet and Granulocyte Transfusion

Janice P. Dutcher

Introduction

A major advance in our ability to treat patients with hematologic malignancies (particularly those with acute leukemia) has been the development of cell component transfusion support during the pancytopenia resulting from remission induction therapy and subsequent intensive consolidation and bone marrow transplantation. The first therapeutic "platelet transfusions" occurred when Duke [1] transfused fresh whole blood in 1910, after noting an association between thrombocytopenia and bleeding and observing that correction of bleeding was associated with an increase in platelet count. However, 50 years were required to develop the technology for separating platelets efficiently from blood and allowing their administration as a separate blood component. In 1963 Freireich et al. [2] demonstrated the clinical effectiveness of platelet transfusions in children with acute leukemia, using platelet-rich plasma as the transfused blood product. The ability to provide platelets as a separate concentrated blood component has substantially improved the hematologic support of leukemia patients and has decreased the incidence of deaths caused by hemorrhage. Further technical advances have improved the quality of this blood product, and careful studies of transfusion practices have led to specific guidelines to optimize platelet transfusion support.

The success of platelet transfusion therapy led to attempts to separate granulocytes as a blood component, to help combat the other major problem in leukemia supportive care—the management of infection. The initial granulocyte transfusions were from patients with untreated chronic myelocytic leukemia and high white blood cell (WBC) counts whose WBCs could more easily be separated from the red cells by gravity [3]. Separating granulocytes from red cells, especially in donors with normal WBC counts, was initially extremely problematic, since little difference in density exists between red cells and granulocytes. Such separation was first attempted by filtration techniques, in which the white cells adhered to filters as whole blood passed through and then the granulocytes were literally hammered off the filters [4]. However, technology quickly advanced for collection of both platelets and granulocytes, particularly with newer cell separator technology, allowing collection of cellular components from whole blood of normal donors [5, 6] with demonstration of clinical efficacy [7].

The use of platelet transfusion support has increased dramatically in the past 20 years, in large part because of more aggressive chemotherapy (for leukemia and solid tumors) and more frequent use of hematopoietic stem cell transplantation (HSCT). In response to the increased usage, as well as concerns about transfusion reactions, transfusion-transmitted infection and alloimmunization, new technological changes have also developed rapidly and are ongoing [8].

Granulocyte transfusion therapy, over the course of the past 25 years, has markedly decreased in frequency. In the late 1970s and early 1980s, granulocyte transfusions were used and studied extensively, to combat neutropenic sepsis in leukemia patients. Two factors impacted on the decline in usage: (1) the development of more effective broad-spectrum antibiotics and the adoption of the policy of empiric initiation of antibiotics for febrile neutropenia and (2) concern that insufficient doses of granulocytes were being obtained from normal donors, even with enhanced technology, to provide sufficient granulocyte numbers for effective anti-infective therapy. However, new approaches to granulocyte procurement have led to greater donor yields, and there appears to be renewed interest in appropriately utilizing granulocyte transfusions. The clinical criteria for such transfusions remain unresponsive sepsis and fungal infections. Ongoing studies are attempting to determine whether the enhanced yields will be sufficient to improve the outcome in these settings.

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Platelet Transfusion Therapy

Platelet Collection

Although platelet-rich plasma (PRP) was shown to diminish or stop bleeding in thrombocytopenic leukemic children, PRP is a large-volume transfusion product and is not practical for general use. The ability to concentrate platelets into a smaller volume of plasma was facilitated by Mourad's [9] discovery that the addition of extra amounts of acid citrate dextrose anticoagulant to the platelet-rich plasma before centrifugation allowed easy resuspension of the platelets, rather than clumping.

Platelets for transfusion are currently prepared from the routine processing of whole blood units (U) into packed red blood cells (pRBCs) and platelets by differential centrifugation (random donor platelets) or by apheresis of single donors (single donor platelets). The method for producing single units of platelets currently utilized in the USA involves separating whole blood into pRBCs and platelet-rich plasma (PRP) [10]. Platelets are subsequently concentrated from PRP, with a minimum of 50 cm^3 of plasma per concentrate. In Europe and in Canada, the separation technique utilizes the hard centrifugation of a whole RBC unit to produce a buffy coat (BC) of WBCs and platelets. This BC is suspended and recentrifuged to yield platelets [11]. With these single-unit processing techniques, 1 U of platelets (from 1 U of blood) contains $0.55–1.1 \times 10^{11}$ platelets. Several units are then pooled to produce a transfusion (4–8 U/transfusion for adults). Units are pooled just prior to releasing a transfusion, and once pooled, they should be transfused within 4 h due to the risks of bacterial contamination once units are opened for pooling. Comparisons of these separation techniques have been made with at least one analysis suggesting less platelet activation with the BC technique [11]. Both methods have substantial leukocyte contamination which requires leukodepletion prior to administration (either prestorage or poststorage—see subsequent discussion).

The other method of collecting platelets involves the use of apheresis machines that can obtain multiple units of platelets from individual donors in a single procedure, lasting 1–2 h. The principle of apheresis, using either intermittent or continuous flow, is similar to that used in the preparation of individual platelet units: separation of blood components by density centrifugation. With an apheresis machine, however, whole blood is withdrawn from the donor into a collection bowl, belt, or chamber within the machine, and the blood is then centrifuged continuously into layers. The platelet or other cellular component is then extracted through a collection port, and the RBCs and plasma and other cellular components are returned to the donor. This process is continued until a sufficient number of platelets are collected. All of the currently utilized apheresis machines have been through numerous updates to maximize platelet yield and to meet the current standards requiring reduced leukocytes in the product [12–16]. Through new technology, donors may frequently supply sufficient platelets for multiple transfusions, during a single apheresis session lasting 1.5 h or less [16]. The clinical practice guidelines of the American Society of Clinical Oncology (ASCO) on platelet transfusions states that either pooled platelets or apheresis platelets for non-alloimmunized recipients are interchangeable [17].

The United States has almost totally adopted apheresis platelets, and has an infrastructure of donor centers designed to utilize this approach. In contrast, Europe and Canada, with National Blood Services, utilize a combination of pooled platelets (the majority) and single donor platelets and have developed infrastructure to support the pooled platelet approach. The technology to support either approach is clearly in place and both utilized leukodepletion techniques. Pros and cons of each approach continue to be debated. Risks of pooled platelets are multiple donor exposures and bacterial contamination from the manipulation, whereas risks of apheresis platelets appear to be increased risk of transfusion-related acute lung injury (TRALI) from antibodies as well as plasma-related adverse events (see section on hazards of platelet transfusions) [18, 19]. The two approaches appear to be equivalent in terms of cost and risk of alloimmunization [17–21].

Apheresis donor-related issues include transient decreases in platelet count that are not significant unless there is supra-frequent donation (in the setting of a necessary HLA-matched donor). Since donors have a normal pre-procedure platelet count, this is rarely a clinical problem. Although the risk of bleeding may be negligible, bleeding times are abnormal with platelet counts of less than 100,000/μL, so platelet counts in individuals who donate frequently must be monitored. Glowitz and Slichter [22] observed serial decreases in donor pre-apheresis counts with frequent donation, but have noted an apparent rebound rise in platelet counts after several donations, suggesting a marrow response with increased production. No adverse effect on donor platelet count was observed with repeated multiunit donation [22]. Additional donor-related problems with apheresis include symptoms of hypovolemia or vasovagal reactions, the discomfort of venipuncture, or mild reactions to citrate anticoagulant (which can be reversed by slowing the flow rate of the procedure). These adverse effects should not prevent the necessary collection of single-donor histocompatible platelets, but must remain a consideration in selecting and repeating procedures in certain donors, particularly when not required for histocompatibility.

Current American Association of Blood Banks (AABB) standards recommend platelet-apheresis donation no more frequently than weekly, or 24 times in a year except in unusual circumstances. These would be decided in conjunction with the treating physician and the approval of the blood bank director [23]. It is recognized that platelets from

a specific histocompatible donor may be essential to the survival of a specific patient and thus donations may exceed the limits indicated by the standards, which has been done safely [22, 24]. The donor must undergo serial clinical and laboratory evaluations by the supervising physician.

A recent report regarding the effect of citrate anticoagulant on bone and mineral metabolism suggests that in regular apheresis platelet or plasma donors, there is a reduction in bone mineral density. In this report, there was a significant reduction in lumbar spine and a less than significant reduction in the femoral neck and total femur. If confirmed, this will need to be addressed for donor safety and to retain regular donors [25].

Commentaries comparing apheresis-donor platelets and single-blood unit-derived platelets consider these products to be interchangeable and suggest that cost factors and donor availability will largely influence the frequency with which each approach is utilized for random donor platelets [8, 15, 17, 18, 26]. However, the recent report on hemovigilance by Daurat suggests an evaluation of recipient toxicity requires further evaluation and will also enter into decision-making [18, 19].

Leukodepletion

Recommended current transfusion practice involves leukodepletion of blood products to remove immunogenic lymphocytes and antigen-presenting cells, to prevent alloimmunization (discussed in detail later). Leukodepletion also reduces the risk of transfusion-transmitted viral disease when the virus is harbored in the leukocytes (see Hazards of Transfusion section later in this chapter). Since lymphocytes are similar in density to platelets, numerous technical difficulties have arisen, and considerable effort has been expended in developing techniques to separate leukocytes from platelets in PRP and in buffy coat-derived platelets.

Leukodepletion of apheresis products relies primarily on the modifications that have been made in the technology of the apheresis machines to reduce leukocyte contamination and retain adequate numbers of platelets per collection [12–16] (Table 54.1).

Single units of platelets derived from PRP or BC can be leukodepleted upon pooling, prior to transfusion, in the blood center, or at the bedside. Leukocyte-adherent filters are utilized for this process. The initial materials utilized were cotton wool and cellulose (Imugard) [27].

Initial studies of nonwoven polyester filters were utilized at bedside or in the blood bank producing adherence of the leukocytes to the filters (Sepacel, Baxter/Fenwal; Pall, Pall Corp) [27–30]. Table 54.1 summarizes the content of the resulting platelet products. Additional investigations of materials and different structures of materials for leukodepletion have been and continue to be conducted [27–31].

Table 54.1 Characteristics of leukodepleted platelet products

Method	Total white blood cells	Platelets $\times 10^{11}$	References
Mechanical apheresis			
Cobe spectra V5-LRS	$<1 \times 10^6$	2.56–7.41	[16]
Fenwal amicus	$<1 \times 10^6$	2.82–7.14	[16]
Cobe V7 turbo	$<1 \times 10^6$	2.80–7.54	[16]
Filtration—6 pooled units			
Imugard IG 500	$0.5–2 \times 10^6$	3.1–4.0	[27]
PALL PL100	$5–6 \times 10^5$	3.5–4.0	[28]
Sepacell PL	$0.1–5 \times 10^6$	3.1–4.0	[29, 30]
Historical centrifugation studies of 6 pooled units	$5–7 \times 10^8$	3.0–4.0	[10]

Many blood centers have also evaluated the prefiltering of products for distribution, and in-line filters have been tested for use during red cell collection and during apheresis. One study has shown no increased risk of bacterial growth in platelet concentrates as a result of prestorage white cell reduction [32]. There has been advocacy for prestorage leukodepletion to diminish cytokine production by leukocytes during storage [33, 34]. Others have demonstrated that poststorage leukodepletion may be more useful in removing complement and reducing reactions [35]. Additional research in leukodepletion technology is attempting to design filters capable of removing prions [36]. These issues point out the complexity of the ongoing clinical investigation of leukodepleted blood products and attempts to define the role and extent of leukodepletion of blood products. Issues not previously discussed include the cost in universal acceptance of this approach [37].

Platelet Storage

The increased demand for platelet transfusions as well as the delay imposed by requirements for multiple pretransfusion infectious agent product screenings has stimulated ongoing research in the technology of platelet storage and methods for evaluation of platelet survival. Many physical factors influence successful platelet storage. These include optimal storage temperature, maintenance of a certain range of plasma pH, suspension in an adequate volume of plasma, proper agitation during storage, and attention to the characteristics of the storage bag [38–45].

Storage conditions for individual platelet units derived from whole blood donations as well as for the storage of apheresis platelet collections have been defined and optimized based on the stability of storage pH and durability of platelet function [39–41]. Current practice based on numerous investigations considers room temperature (22–24 °C) storage as optimal for platelets stored in plasma [39, 40, 42, 43]. Despite the increased metabolic activity that occurs in

platelets stored at 22 °C, significantly better poststorage platelet viability at this temperature (compared with platelet concentrates stored at 4 °C) has been confirmed by studies of transfusion response after storage at the two different temperatures [39, 40, 42].

The storage bag characteristics have also been shown to be important in the maintenance of platelet viability [38, 39]. The development of new plastics and plasticizers and the production of larger and thinner bags have established a more stable storage environment, making possible extended storage of platelet concentrates and single-donor apheresis platelets [43–45]. Storage bag qualities help to regulate oxygen and carbon dioxide exchange across the bag, which in turn regulates pH. The maintenance of plasma pH above 6.0 during storage preserves cell viability, although a very high pH can lead to irreversible platelet clumping [38–42]. Entry of adequate oxygen permits the maintenance of oxidative metabolism in platelets stored at room temperature, thus suppressing glycolysis (with its production of lactic acid) and preventing a fall in pH level. The egress of carbon dioxide across the bag surface also helps to maintain pH. The newer plastic bags provide increased oxygen transfer compared with the first-generation bags and have allowed stable, prolonged, storage with viability demonstrated after 5 or more days [38–44].

Agitation during storage of platelets is also important, since it prevents platelet "packing" and is believed to facilitate gas exchange through the storage bag [45, 46]. With the use of the permeable storage bags, it seems that the mode of agitation is less critical to platelet viability, and a variety of platelet rotators appear to be suitable for maintaining function [45, 46].

These conditions have yielded functional platelets, despite storage for 5 days under these conditions. Most recently, modifications in storage systems have demonstrated equivalent outcome for 7 day storage in plasma and this has led to regulatory approval [47]. This approach requires pre-infusion bacterial testing [48].

However, even with the many improvements in technology, the collection and storage of platelets leads to platelet activation with a subsequent "storage lesion" [49, 50]. Platelets appear to be activated throughout the process of collection, processing, and storage, demonstrating metabolic and functional signs of activation [49]. Thus, stored platelets show less sensitivity to aggregating agents [51] and release of increased amounts of platelet-specific proteins such as β-thromboglobulin [52, 53] with membrane expression of activation proteins such as P-selectin (CD62P) and glycoprotein IIb/IIIA [53–55]. Stored platelets have reduced platelet survival corresponding to the duration of storage [49, 50]. Snyder et al. have delineated multiple factors that impact platelet storage viability (Table 54.2) [8]. Research in storage technology has included the inactivation of platelets during storage [56, 57] and the use of non-plasma-buffered storage solutions as well as additive solutions with plasma reduction [58–63]. Evaluation of quality of such platelets is ongoing. Additionally, pathogen reduction storage systems are undergoing evaluation [64–66].

Table 54.2 Factors contributing to platelet storage lesion

During collection of platelets
Type of anticoagulant
Sheer effects from blood drawing—activation by catheter
Time between whole blood collection and separation
Temperature
During storage of platelets
Storage duration
Temperature of storage
Mode of agitation
Storage container (gas exchange)
Irradiation
Leukodepletion filter (activation)
Cryopreservation

Adapted with permission from Perrotta and Snyder [8]

Platelet Cryopreservation

The technology for long-term frozen platelet storage has proved clinically helpful in the management of alloimmunized patients at the University of Maryland and Albert Einstein Cancer Centers [67, 68], and in Europe [69]. During recovery from chemotherapy-induced aplasia and achievement of remission, such patients often have rapidly rising platelet counts, and plateletpheresis during this period often provides a high yield of platelets for cryopreservation. Both autologous and HLA-typed platelets have been frozen for subsequent use in alloimmunized patients. Initial cryopreservation techniques using either dimethyl sulfoxide (DMSO) [70, 71] or glycerol [72, 73] have been described, but to date only the DMSO methodology has been reproducible enough for general usage. This technology calls for a slow infusion of DMSO into PRP to a concentration of 5%. This is similar to the methodology used for marrow and stem cell cryopreservation. Recoveries are 50–70% of fresh platelets, and satisfactory hemostatic results are documented; occasional alloimmunized patients are supported solely by cryopreserved autologous platelets [67, 68, 74].

Successful transfusion of platelets that have been stored at liquid nitrogen temperatures (−120 °C) for 3 years or more has been documented [74]. This freezing methodology is also potentially applicable to collection and storage of platelets with specific HLA types or for the maintenance of emergency supplies. One report suggests that the addition of a small percentage of epinephrine to the DMSO cryopreservation solution may enhance the quality of the cryopreserved platelets, by increasing post-thawing function [75]. Finally, recent studies to attempt to improve on the frozen platelet

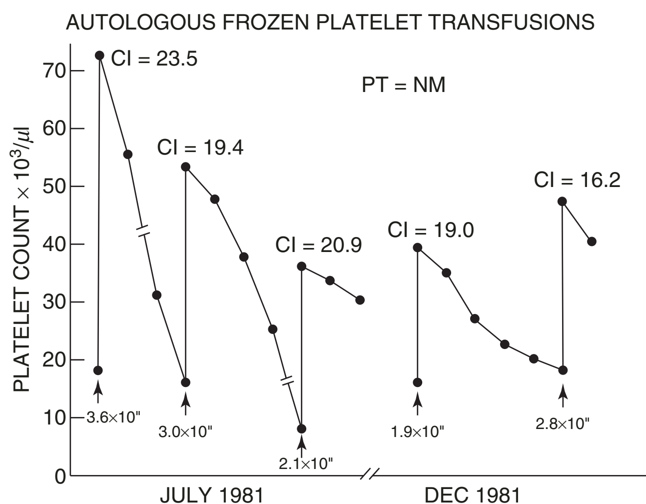

Fig. 54.1 Clinical effectiveness of autologous frozen platelets in an alloimmunized patient. This patient had episodes of mild bleeding, which responded to transfusions of autologous frozen platelet transfusions. The increments following transfusion were consistently good. *CI* corrected increment

methodology have confirmed prior data that despite the freezing injury, the platelets retain function and are able to aggregate, and that there is an in vivo improvement in function once the previously frozen platelets circulate [76].

Figure 54.1 shows the consistently good response to transfusion of autologous frozen platelets in a patient who was alloimmunized and refractory to transfusion from random donors. The patient received multiple courses of intensive postremission chemotherapy, each requiring transfusion support. As shown, the patient was an excellent and readily available source of platelets for herself.

Although the above data are historical, at that time, these approaches produced evidence of major benefit, particularly for alloimmunized patients. Now, there has been a resurgence of interest in cryopreservation of platelets which has paralleled the increase in demand for platelet transfusion support. Evaluations of functionality are being performed and an initial phase I trial in healthy subjects demonstrated impressive survival of cryopreserved platelets [77]. Others are further investigating the hemostatic activity after cryopreservation and have demonstrated such activity associated with formation of platelet microparticles [78–81]. Other cryopreservatives continue to be studied as this approach gains more interest to provide further availability of this component [82].

Therapeutic and Prophylactic Platelet Transfusions

A direct relationship exists between the platelet count and the bleeding time, which is used to assess platelet function in vivo [83]. The bleeding time will begin to lengthen as the platelet count falls to less than 100,000/μL, although clinically apparent bleeding problems may not develop until the platelet count is substantially lower (less than 20,000/μL). Early studies of patients with acute leukemia described the quantitative relationship between low platelet counts, duration of thrombocytopenia, and episodes of serious bleeding [2, 84] (see Chaps. 21 and 59). It was subsequently demonstrated that platelet transfusions could diminish the severity of bleeding in thrombocytopenic leukemia patients with ongoing hemorrhage. The effectiveness of therapeutic platelets transfusions is well documented [2, 84]. This observation led to the suggestion that platelets given prophylactically for low platelet counts might therefore help prevent bleeding. Since life-threatening hemorrhage can develop rapidly in the absence of prior signs of minor bleeding, particularly in patients with concurrent clinical problems, prophylactic platelet transfusion given for a platelet count of 10,000–20,000/μL is usually the practice, rather than waiting for bleeding to occur, and this remains a part of clinical guidelines for management of therapy-induced hypoproliferative thrombocytopenia [17, 85, 86]. This practice continues to be refined as will be discussed.

It has become apparent that thrombocytopenic patients vary greatly in their likelihood to develop serious bleeding as well as in their ability to respond to random donor platelet transfusions. Therefore, assessment of the clinical setting should determine whether prophylactic platelet transfusions are indicated [17, 85–87].

Many patients with chronic hematologic disorders who maintain platelet counts of less than 20,000/μL or even less than 10,000/μL have no or minimal bleeding. In such patients, a low but stable platelet count is tolerated without transfusion unless other clinical factors are also present, such as infection or a bleeding site [17, 85, 87].

The other example is in chemotherapy-treated patients with solid tumors who are not treated at doses designed to achieve marrow aplasia and who have functional marrow reserve. A review of more than 1200 such patients who experienced transient thrombocytopenia (less than 20,000/μL for a median of 2 days) showed no bleeding as a result of thrombocytopenia alone [88]. Those who did experience hemorrhage demonstrated the other clinical complications that have been shown to predispose to bleeding during thrombocytopenia, such as mucositis, a bleeding necrotic tumor, fever, and/or sepsis; bleeding frequently occurred at platelet counts above 20,000/μL [88].

Elting and a group from MD Anderson Cancer Center have also evaluated the risk of bleeding in patients with lymphoma and solid tumors who are being treated [89]. They have derived a bleeding risk index, with clinical factors similar to those in leukemia, which predicts for bleeding in this patient group, more than does a specific platelet count threshold [89].

Table 54.3 Clinical conditions affecting platelet transfusion outcome

Clinical factor[a]	Shorten 1-h count	Shorten 24-h count
Fever	No	±
Infection, controlled	No	±
Pre-platelet count <10,000	No	+
Bleeding: ooze	No	±
Daily RBC transfusion	±	+
Coagulopathy: DIC	±	+
Palpable splenomegaly[b]	±	+
Sepsis[b]	±	+
Hypersplenism	+	+
Anti-HLA antibodies	+	+
Amphotericin therapy[b]	+	+
Bone marrow transplantation[b]	+	+

[a]Data from Daly et al. [90] and from Slichter [87]
[b]Data from Bishop et al. [91]

Table 54.4 Factors affecting posttransfusion platelet increments and refractoriness

Patient factors
Reduced response and/or refractory to transfusions
More than two prior pregnancies
Presence of lymphocytotoxic antibody (LCTAb)
Male gender
Bleeding
Fever, infection
Increased body surface area/weight
Increasing number of platelet transfusions
Heparin therapy
Reduced platelet increment, additional factors
Splenomegaly
Disseminated intravascular coagulopathy
Amphotericin B therapy
Improved platelet increment response
Splenectomy
Increased patient age
Improved response to refractoriness
Increased platelet dose
Filtered apheresis platelets
Platelet factors
Improved response
ABO compatible
Storage <48 h
Large doses of platelets
Decreased response
UVB or Gamma irradiation[a]

Data from Slichter SJ, Davis K, Enright H et al. Factors affecting posttransfusion platelet increments, platelet refractoriness, and platelet transfusion intervals in thrombocytopenic patients. Blood 2005; 105:4106–4114
[a]In alloimmunized, LCTAb + patients, UVB-irradiated platelets had longer survival than other products

By contrast, patients with acute leukemia and those undergoing highly intensive chemotherapy for other hematologic malignancies, whose treatment usually leads to marrow aplasia and in whom marrow regeneration may not be expected for a prolonged time period, even weeks, are usually treated prophylactically with platelet transfusions for expected prolonged severe thrombocytopenia. These patients frequently develop concurrent clinical conditions that add to the risk of thrombocytopenic bleeding: mucositis, fever, infection, and many of the other clinical complications listed in Table 54.3 [87, 90, 91]. Prophylactic platelet transfusions given to those patients who respond to transfusions with a posttransfusion increment are indicated in this setting [17, 87, 91].

The demand for platelet support for patients with hematologic malignancies has greatly increased, given the ability to successfully treat even frail patients and the increasing numbers of hematopoietic stem cell transplants (HSCT) (Chap 57), both autologous and allogeneic, and a spectrum of conditioning approaches. This high demand has prompted investigations to optimally conserve this important resource. This includes studies of the threshold platelet count for transfusion, optimization of dose of platelets per transfusion and patient size, and most recently, evaluations of prophylactic platelet transfusions versus therapeutic transfusions for bleeding episodes. Additionally, the ability to nearly universally leukodeplete all blood products has greatly reduced the incidence of transfusion-induced alloimmunization (TRAP Study) lowering the demand for specially matched platelets [92]. The TRAP study also provided a contemporary listing of patient and platelet factors associated with reduced transfusion responsiveness and refractoriness (Table 54.4).

With respect to transfusion threshold, the platelet count trigger to initiate prophylactic platelet transfusion has been a matter of debate, and the count plus the clinical setting seems the best approach. Three randomized trials have evaluated using a platelet count trigger of 10,000/μL vs. 20,000/μL in patients with acute leukemia [93–95]. In all three studies, there was no difference in major bleeding [93–95]; however, only one found no difference in red blood cell transfusion requirements [93], and only one found that fewer platelet transfusions were administered [95].

A statistical analysis of the risk of bleeding in the Rebulla study determined that the risk factors for mild bleeding were increased body temperature and decreased platelet count [96]. Risk factors for clinically significant bleeding were grade 1 bleeding the previous day, low platelet count, and fever. Thus, as noted in many prior studies, clinical factors in the setting of profound thrombocytopenia markedly enhance the risk of bleeding (Table 54.3) [17, 91, 96]. Additional studies have demonstrated maintenance of endothelial integrity with platelet counts of ≥5000/μL [97].

In addition to the conditions discussed previously, patients who demonstrate a rapidly falling platelet count appear to be predisposed to bleeding. Patients with hyperleukocytosis, particularly consisting of blasts, are also at greater risk and should receive platelet transfusions if they are thrombocytopenic. Finally, patients with coagulopathy, as is common in patients with acute promyelocytic leukemia (discussed in detail in Chap. 21), are at much greater risk of bleeding from their ongoing coagulopathy. They have been managed with heparin therapy, although this need may diminish in the future, and they often require frequent platelet transfusions, and coagulation factors [98, 99]. Patients with liver dysfunction and decreased production of clotting factors are also at greater risk for bleeding, from the combined effect of both coagulopathy and thrombocytopenia. More frequent platelet transfusion may be necessary in such patients since they are very likely to have ongoing bleeding. Hematologic malignancy patients with other types of coagulopathies undergoing treatment may also require intensive platelet support (Chap 59). Patients who are refractory to platelet transfusion in these acute settings pose a difficult dilemma, and this will be discussed subsequently. In general, if there is no platelet product that yields a posttransfusion increment, careful observation without transfusion (unless active bleeding is present) is the recommended course of action. This is stressful for staff and patient but is unusually manageable and is the safest course in the long run.

The increased availability of platelet transfusions has also permitted the safe performance of diagnostic and therapeutic procedures in thrombocytopenic patients. Platelets can be transfused just before or during invasive diagnostic procedures, such as bronchoscopy or endoscopy, and may permit the safe performance of brushings or biopsy. Therapeutic procedures such as the placement of permanent central intravenous lines (Hickman catheters), the drainage of infected paranasal sinuses, tooth extractions, or even emergency major surgery can be safely accomplished in thrombocytopenic patients with the use of platelet transfusions [100, 101]. Although no count threshold has been proven, we and others have observed that platelet counts of more than 50,000/μL provide a margin of safety for invasive procedures [100, 101], and the AABB has recently provided guidance for platelet transfusion in these settings [85]. It is very important to document a satisfactory posttransfusion count increment before initiating the procedure, to be confident that hemorrhage can be controlled during the procedure. It is equally important to maintain the platelet count at 50,000/μL or more for several days immediately following an invasive procedure, particularly after a biopsy or surgery.

To some degree, the debate regarding platelet count trigger for prophylactic platelet transfusion has been resolved with the randomized trials and the definitions of at risk clinical situations. However, the other debate among transfusion support providers is the dose of platelets necessary for a successful prophylactic transfusion. The goal is to provide sufficient patient support with the least exposure to risks of transfusion [infections, transfusion-related acute lung injury (TRALI)], and with the managed costs and preservation of platelet availability.

Two randomized, multicenter trials evaluating dose of platelets have been reported [102, 103]. The SToP trial was conducted at centers in Canada, the USA, and Europe, and compared standard-dose platelet transfusions to transfusions of 50% less platelets [102]. Transfusions were a fixed dose per product transfused. In this study, there was more bleeding in the low dose arm, including more grade 4 bleeding, which led to the trial being stopped by the Data Safety and Monitoring Committee [102]. It may be that the fixed dose was part of the issue, although there was an upper weight limit, since in the other study described next, platelets were dosed based on BSA. In the PLADO study in the USA, there were three dose levels of platelets: $1.1 \times 10^{11}/m^2$, $2.2 \times 10^{11}/m^2$, and $4.4 \times 10^{11}/m^2$ [103]. The transfusion trigger was a platelet count of 10,000/μL and this was adhered to in 90–94% of patient days and was similar in all three transfusion groups. The percentage of patients having grade 2 or higher bleeding was not significantly different, 71%, 69%, and 70% in low, medium, and high groups, respectively, and the only death from bleeding was in the high-dose group (pulmonary hemorrhage). In this study, the percentage of patients with grade 2 or higher bleeding was significantly greater among those undergoing chemotherapy (73%) or allogeneic stem cell transplant (SCT) (79%) compared to those undergoing autologous or syngeneic SCT (57%). However, platelet dose did not demonstrate an effect on bleeding in any of these groups. There was no difference in grade 4 bleeding among these different treatment groups [103]. Among these three dose groups, the low dose significantly reduced the quantity of platelets transfused, although with more transfusions given. Equivalent numbers of transfusions were given in the medium- and high-dose groups. This study suggests that a strategy of low-medium dose per BSA of platelets could reduce the number of platelets transfused, and preserve the resource that is increasingly being utilized, and maintain safe management of patients undergoing treatment for hematologic malignancies and HSCT [103].

Two studies were reported evaluating the timing of platelet transfusions for patients with therapy-induced hypoproliferative thrombocytopenia [104, 105]. These studies compared the incidence of World Health Organization (WHO) grade 2 or higher bleeding (Table 54.5) between groups of patients who received transfusions either prophylactically at the prespecified morning platelet count of 10×10^9 per liter, or therapeutically for clinical evidence of bleeding, or before invasive procedures or as decided by the treating physician. Patients who had prestudy episodes or evidence of bleeding were

Table 54.5 World Health Organization bleeding scale grade 2–4

Site of bleeding	Grade 2 Evidence of bleeding
Oral/Nasal	Epistaxis or oral bleeding >30 min per 24 h period
Skin/soft tissue	Purpura >1 in.
Musculoskeletal	Spontaneous hematoma in deeper tissues
	Joint bleeding
Gastrointestinal	Melanotic stool; Hematochezia—visible blood
	Hematemesis—visible blood
Genitourinary	Gross (visible) hematuria
	Abnormal, excessive vaginal bleeding
Pulmonary	Hemoptysis—visible blood
CNS	Retinal bleeding without visual impairment
	Microscopic RBC on lumbar puncture—nontraumatic tap
Invasive sites	Bleeding—i.e., venipuncture; IV site >1 h per 24 h
Grade 3	Any bleeding requiring RBC transfusion over routine transfusion needs
	Any bleeding associated with moderate hemodynamic instability and requiring RBC transfusion
Grade 4	Any bleeding associated with severe hemodynamic instability; fatal bleeding; CNS bleeding on imaging study; retinal hemorrhage with visual impairment; CNS symptoms with nontraumatic lumbar puncture

Ref. [106]

excluded, as were patients with promyelocytic leukemia. In the Wandt et al. study, more than 50% of patients were undergoing autologous SCT, and in the Stanworth study, 70% were undergoing autologous SCT [104, 105]. As can be seen in Table 54.5, Grade 2 bleeding is clinically evident bleeding and the distinction between grade 2 and grade 3 is that grade 3 bleeding initiates RBC transfusion specifically for bleeding above and beyond routine red blood cell support for hemoglobin level [106]. As stated in an editorial analysis by Slichter, in both studies, bleeding rates were higher in the no-prophylaxis groups, and the time to onset of bleeding was shorter, and the number of days of bleeding was longer in the no-prophylaxis groups than in the prophylaxis groups [107]. Also of concern, in the Stanworth study there were 6 episodes of central nervous system bleeding among the no-prophylaxis AML patients, 2 of which were fatal [105].

Subsequent subset analyses of the Stanworth study have compared the risk and degree of bleeding among the patients undergoing autologous SCT with those with de novo AML or undergoing allogeneic SCT, and have found a significantly shorter time to first bleeding episode, and increased duration of grade 2–4 bleeding among those with AML/alloHSCT compared to those undergoing autologous SCT [108, 109]. A large number of patients in the autologous SCT group in this study required no platelet transfusions [108, 109]. Wandt et al. have also reported the option of only therapeutic platelet transfusions in the setting of autologous SCT, however, they still report a greater incidence and degree of bleeding in the no-prophylaxis group [110, 111]. Campbell et al. reported a cost analysis of the Stanworth trial and concluded that it was unclear whether a no-prophylaxis policy saves costs overall, since platelets are a small percentage of the total cost of the treatment of de novo AML or of transplant patients [109, 112]. Similarly, Slichter addressed the issue of reducing transfusion exposure, and noted that even with increased platelet transfusions in the prophylactic transfusion group, there were fewer RBC transfusions, therefore producing equivalent donor exposures to those receiving less platelets but more RBC transfusions [107, 113]. In summary, the prophylactic approach to platelet transfusion remains the standard of care in the treatment of patients with therapy-induced hypoproliferative thrombocytopenia and is recommended by guidelines from the AABB and ASCO [17, 85].

Other important data regarding bleeding risk even in the setting of prophylactic platelet transfusion has come from subset analysis of the PLADO trial, which reported that bleeding risks are higher in children included in that study, compared to adults, and significantly high in those undergoing autologous SCT [114]. This was observed at a wide range of morning platelet counts [114]. This may in part reflect the intensity of chemotherapy regimens utilized in children, but may also be evidence of differing vascular physiology in children and suggests that studies of adults may not be generalizable to pediatric patients [107, 114, 115].

Assessment of Transfusion Response

The goal of therapeutic platelet transfusions is to stop active bleeding, which is usually easily assessed clinically. The effectiveness of prophylactic platelet transfusions is more difficult to evaluate, although as noted decreased episodes of bleeding are measurable. Bleeding times, although accurately reflecting platelet function in vivo, are not usually done in granulocytopenic patients because of the risk of infection and because achievement of a platelet count of 100,000/μL is required to normalize the bleeding time [83]. Therefore, response to transfusion is monitored by observing the increase in platelet count shortly after transfusion (i.e., platelet recovery). The increment achieved depends on the size of the recipient and on the number of platelets given. As stated above, the PLADO trial evaluated 3 levels of platelets, $1.1 \times 10^{11}/m^2$, $2.2 \times 10^{11}/m^2$, and $4.4 \times 10^{11}/m^2$ and observed good control [103].

The survival (duration of time circulating) of transfused platelets can be followed by obtaining daily platelet counts following a platelet transfusion. In a patient who is truly aplastic and not producing platelets, the half-life of the transfused platelets can be calculated. Platelet survival will be

shortened by a number of clinical factors, which, in turn, increase the requirement for platelets. These factors are similar to those that increase the risk of bleeding, and include active bleeding, fever, infection, coagulopathy, and hepatosplenomegaly. Platelet survival is also shortened by the development of alloimmunization to histocompatibility antigens, producing immunologic destruction of the transfused platelets. To differentiate alloimmunization from other forms of platelet destruction, platelet recovery after a transfusion (which is diminished by alloimmunization) may be helpful in stable patients. The posttransfusion recovery is assessed by calculating the corrected count increment:

$$\text{Corrected count increment}\left(\text{CCI}\right)=\frac{\text{Observed increment }\left(\text{count}/\mu L\right)\times\text{ body surface area }\left(\text{m}^2\right)}{\text{Number of platelets transfused }\times 10^{11}}.$$

For example, an improvement in count from 20,000 to 60,000/μL after 4×10^{11} platelets when given to a 2.0 m^2 individual produces a corrected count increment of 20,000/μL.

After the transfusion of fresh or properly stored random donor platelets to a clinically stable adult patient of average size, the absolute count increment at 1 h should be 6000–8000/μL/U of platelet transfused, with a CCI of 10,000–20,000/μL. The hallmark of the clinical diagnosis of alloimmunization is a failure to achieve an adequate platelet count increment at 1 h posttransfusion [90] (for practical purposes, a 10-min posttransfusion count may be equally helpful) [116]. Other clinical factors may contribute to the rapid utilization of platelets, however, and may reduce the posttransfusion count (Tables 54.3 and 54.4).

Bishop et al. [91, 117] performed a multivariate analysis of clinical factors influencing the efficacy of pooled platelet transfusion as determined by the 1-h posttransfusion platelet CCI. This study identified six major clinical factors that affected 1-h posttransfusion platelet counts. Whereas splenectomy resulted in improved increments, several factors had a negative effect on transfusion outcome: palpable splenomegaly, bone marrow transplantation, disseminated intravascular coagulation, amphotericin B therapy (which may reflect the degree of illness of a given patient, who may have fever and infection), and anti-HLA antigen-directed antibodies [91, 117]. This group performed a similar evaluation of the effect of clinical factors on the 20-h platelet count (platelet survival) and again found that the clinical situation had a significant impact [117]. The combination of all these factors makes confirmation of the diagnosis of alloimmunization difficult in rather complex clinical cases, and refractoriness to transfusion without development of antibodies appears to reflect the multitude of clinical factors impinging on posttransfusion platelet counts [91, 117–119].

In addition, Hanson and Slichter [120] demonstrated a reduced platelet life span in severely thrombocytopenic patients, presumably due to utilization of platelets for support of vascular integrity. In this study using autologous platelets, platelet life span was only modestly shortened when patients with platelet counts in the 50,000/μL to 100,000/μL range (7.0 ± 1.5 days, 9.6 ± 0.6 days, respectively) were transfused, but was markedly shortened in severely thrombocytopenic patients (less than 50,000/μL) (5.1 ± 1.9 days) [120]. Recovery of autologous platelets was also diminished in patients with platelet counts less than 50,000/μL (50 ± 22%) compared with that in patients who had counts more than 50,000/μL (74 ± 15%).

The controversy regarding the effectiveness of platelet transfusion mismatched for ABO continues to be discussed. An early study indicated decreased platelet survival with ABO-mismatched platelet transfusions [121]. This study evaluated paired ABO-matched and ABO-mismatched random donor platelet transfusions and demonstrated a detrimental effect of ABO mismatch on platelet transfusion outcome [121]. Although the initial platelet transfusion was not always affected by ABH incompatibility, results of subsequent mismatched transfusions deteriorated, perhaps because of an increase in ABH antibody titers [121].

Nevertheless, platelets can successfully be transfused across ABH groups. Clinical complications or transfusion reactions are only rarely associated with ABH-mismatched platelet transfusions. A retrospective systematic review of the literature on transfusion of ABO-identical vs. nonidentical platelets evaluated both transfusion and patient outcome [122]. There was a consistently higher platelet increment with the use of ABO-identical platelet transfusions, but there was no consistent pattern of negative clinical impact on patient survival, bleeding, or transfusion reaction reported [122]. In a secondary analysis of platelet transfusion characteristics in the PLADO dose study, generally higher platelet increments were obtained with ABO-identical platelets and the interval between transfusions was longer with ABO-matched platelets, but there was no demonstrable negative effect on incidence of bleeding from utilization of ABO-mismatched platelet transfusion [123]. In contrast, Julmy et al. have presented data in children which demonstrate considerably poorer efficacy of ABO major-mismatched platelets compared to ABO identical platelets [124]. This is particularly apparent in platelets of subgroup A_1 significantly expressing A antigen, and these were rapidly removed from circulation in recipients who were group O or group B. Those donor platelets that were subgroup A_2, expressing no detectible antigen, were successfully transfused. Therefore, it is still a consideration to evaluate ABO-identical platelet

transfusions in patients who have become refractory and who have received ABH-mismatched platelets.

Sensitive techniques have demonstrated the presence of intrinsic H substance on platelet membranes with expression of ABH antigens corresponding to the RBC phenotype, suggesting the presence of intrinsic and extrinsic antigenic substance residing on platelets [125, 126]. The report by Julmy et al. demonstrates the potential clinical impact [124]. Additionally, DeLelys et al. have demonstrated wide variability of expression of blood group A and H on platelets, but in a predictable pattern, related to genotype [127].

Alloimmunization

Alloimmunization is the presence or development of antibodies (primarily to histocompatibility [HLA] antigens) which may lead to immune-mediated refractoriness to platelet transfusions. Patients with exposure to blood products prior to therapy for their hematologic malignancy or women who have had prior pregnancies are the most likely to have preexisting alloimmunization. Prior to the routine use of leukodepleted blood products in the supportive care of hematologic malignancy patients, acquired alloimmunization was a major factor in the development of refractoriness to platelet transfusions and led to hemorrhagic deaths. However, with the frequent use of leukodepleted blood products, the new development of allo-antibodies is much less frequent, as demonstrated in the TRAP study [92]. Whereas patients with preexisting immunization may have persistent allo-antibodies, requiring specialized HLA-matched or crossmatched platelet support, those with treatment-associated acquired antibodies may not have persistent antibodies [128, 129]. In the TRAP study, factors associated with development of antibodies were prior pregnancies and a broad spectrum of antigen reactivity in a lymphocytotoxicity panel [129]. In the PLADO trial, prior pregnancy, chemotherapy only compared to progenitor cell transplant, and low platelet dose were all associated with higher rates of alloimmunization [130].

Additionally there is a substantial group of patients who do not become alloimmunized during remission induction therapy, perhaps a result of therapy/disease mediated immunosuppression [92, 131–133]. These data are derived from an era prior to significant leukodepletion of blood products, and contemporary studies demonstrate an even lower rate of alloimmunization [129, 130]. Most importantly, there was no evidence of a dose response between the numbers of lymphocyte-containing platelet transfusions and the development of alloimmunization [132, 133].

Specifically, in the study of patients with acute myeloid leukemia (AML) by Dutcher et al. [132], nonalloimmunized patients were evaluated during remission induction therapy (the first 8 weeks of treatment) for the development of alloimmunization, assessed by response to random donor platelet transfusion and the development of lymphocytotoxic antibodies. All patients received non-leukoreduced random donor platelet transfusion (mean, seven transfusions; range, two to more than ten), given prophylactically and therapeutically. Of the patients not previously alloimmunized, 40% became alloimmunized, some after as few as two transfusions (Fig. 54.2). By contrast, 60% of the patients did not become alloimmunized, even after multiple platelet transfusions. Except for previous pregnancies and blood transfusions, no predisposing factors were predictive of the

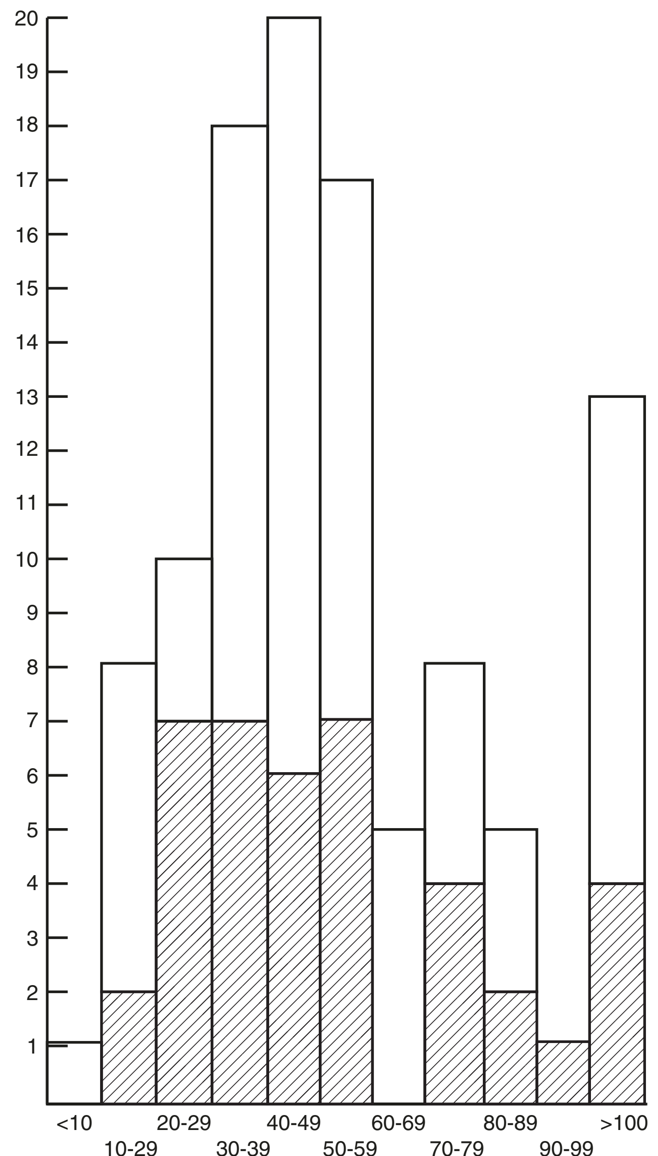

Fig. 54.2 Number of units of platelets administered per patient during induction therapy in patients with acute leukemia. There is no relationship between the number of units given and the rate of alloimmunization. *Cross-hatching*: patients who became alloimmunized (Reproduced with permission from Dutcher et al. [132])

development of alloimmunization. Since a minimum of two platelet transfusions (and usually more) is needed for support through the remission induction phase of AML treatment, no rationale exists for restricting the number of platelet transfusions specifically to prevent alloimmunization [132]. No dose–response phenomenon occurs at a clinically manageable level, and the development of alloimmunization appears to depend on the immunologic responsiveness of the recipient. Long-term follow-up showed that those who did not develop antibodies during initial anti-leukemic therapy were unlikely to develop antibodies during subsequent consolidation or maintenance therapy [131].

Much of the body of work evaluating the impact of anti-HLA antibodies on platelet transfusion results utilized panels of lymphocytes expressing known antigens and assessed the percentages of antibodies to the panel (lymphocytotoxic antibodies) in the sera of recipients [90, 134–136]. Several studies demonstrated correlation of the presence of these antibodies with lower posttransfusion count increments [90, 134–136]. Platelet transfusion support was then achieved utilizing selection from large pools of HLA-typed donors and matched at HLA-A and HLA-B loci [137]. The demonstration of limited expression of certain HLA antigens on platelets, as well as cross-reactive groups facilitated donor effectiveness even when not perfectly matched [138–140]. This is demonstrated in Fig. 54.3 in which a patient and donors are mismatched for HLA-B7, HLA-B12, and HLA-B44 and yet there are successful transfusion outcomes (transfusion #2,# 4, #6, # 7) similar to the perfectly matched donor (#3). In contrast, when mismatched at a strongly expressed antigen (#5), the poor result is similar to that of random donor platelets (#1). These cross-reactive antigen groups reflect differences in public and private epitopes and the use of this strategy is described as utilizing cross-reactive groups (CREGs) [141]. Further discussion of newer strategies for matching of platelets for alloimmunized patients will be presented subsequently.

If appropriately matched donors cannot be located for refractory alloimmunized patients, it is inadvisable to continue to administer random donor platelets prophylactically because the risks and discomforts of such transfusions outweigh the benefits. However, significant bleeding in these patients can sometimes be managed by infusions of massive amounts of unmatched platelets [119], although heavily sensitized patients even then do not obtain count increments and continue to bleed. The possible mechanisms leading to response with this approach may include the accidental inclusion of partially matched platelets in the random pooled platelets or a temporary reduction in antibody titer by the large amount of infused antigen, enabling some of the transfused platelets to survive.

As noted above, it has been demonstrated that some patients lose their allo-antibodies over time, particularly those who develop these antibodies while on treatment [128, 129, 131]. In certain cases, no recent antigenic challenge has occurred, which would explain the decline in lymphocytotoxic antibody levels, and a rapid, anamnestic response to rechallenge is often seen. However, in an early study of 234 patients who had developed lymphocytotoxic antibodies at some time in their clinical course, 70 (30%) had significant declines in antibody level [128]. Among 35 evaluable patients, previously alloimmunized, who then lost their antibodies and were rechallenged with random donor platelets, 34 patients when rechallenged responded with good transfusion outcomes to random platelets for 2 weeks to 36 months [128]. Twenty-one patients failed to regenerate lymphocytotoxic antibodies despite repeated transfusions [128]. Again, in the TRAP study, 145 patients became antibody positive, and 81 (56%) subsequently became antibody negative [129].

Over the past decade, technology has evolved to identify the numerous epitopes or alleles that are components of the individual HLA antigens (see Chapter 56). This information has been particularly important in advancing the field of mismatched stem cell transplantation, but is also being utilized

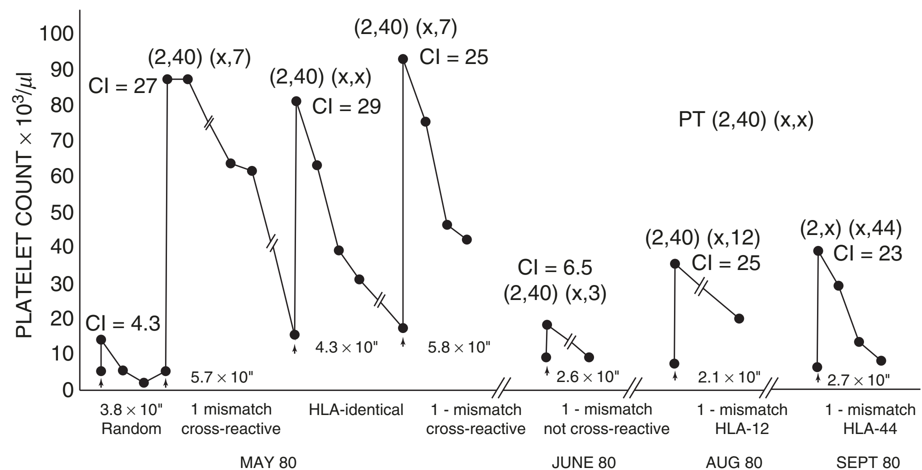

Fig. 54.3 Clinical course of this alloimmunized patient demonstrates the value of selectively mismatched platelets when the antigens are cross-reactive with recipient antigens (transfusions #2 and 4). Platelets mismatched at HLA-44 were also clinically effective (transfusion #7). *Parentheses*, HLA phenotypes; *CI* corrected increment

to define optimal platelet donor/recipient matches. Duquesnoy et al. have developed a structurally based matching algorithm based on amino acid triplets and subsequently epitopes called eplets to further define the HLA phenotypes [142]. This HLA-matchmaker program has been utilized and reported on, and a prospective trial is underway [142–144]. Additionally, the refined exposition of the HLA structure has led to an understanding that allo-antibodies have different specificities among these epitopes, with differing impact on transfusion outcome. Petz et al. recommended identifying donors based on recipient antibody profile [145]. Technology has been developed to provide more sensitive anti-HLA antibody detection, with techniques such as enzyme-linked immunosorbent assays (ELISA) and mini-flow bead platform binding (Luminex Corp) [146–150]. The latter was initially directed toward solid organ transplantation, but more centers with solid and stem cell transplant programs are using antibody profiles to help direct platelet transfusions for alloimmunized recipients [148, 149]. The sensitivity of these approaches are relevant to organ transplantation, but may be overly sensitive in the setting of platelet transfusion, and Jackman et al. has reported that low level HLA-antibodies, as determined by modern bead-based technology, did not predict platelet transfusion failure in the TRAP study [151]. Antibody cutoffs continue to be defined and likely have different implications in transplantation and transfusion.

There is continued interest in utilizing cross-matching tests with the platelet as the target cell. The original approach was to use stored donor platelets from potential donors, to be cross-matched with recipient serum prior to transfusion [152, 153]. Moroff et al. [152] evaluated comparative transfusion data in patients known to be refractory to random donor platelets, choosing donors by HLA matching or by platelet cross-matching using three different methods. They found comparable percentages of successful transfusions using either HLA-selected or cross-matched-compatible donor selection. In another early study, both HLA-compatible and cross-match-compatible methods were effective in providing single-donor platelets and the authors concluded that these were independently important selection methodologies [153].

More contemporary reports of cross-match-compatible platelet transfusions for refractory patients, utilizing newer methodology, provide interesting insights [154–157]. In one report, cross-match was effective in providing successful transfusions except in patients with broad reactivity, and this group suggests utilizing broad reactivity to trigger a workup for HLA-matched platelets [154]. Another report utilizing a modified antigen capture ELISA cross-matching technique appeared to be quite successful in selecting transfusions [155], and similarly, reports utilizing a solid-phase red cell adherence technique was reported to be successful [156, 157]. However, Rioux-Masse et al. compared utilization of cross-matched or HLA-matched platelets from a blood center, and found unsatisfactory results with both approaches [158]. Other clinical factors affecting platelet transfusion outcome were suspected, but not identified, and the numbers of transfusions and patients were small [158].

All methods of donor selection have their proponents in the blood bank and transplantation community, based on technology available, patient needs, donor availability, and cost in time and expense.

Table 54.6 provides an algorithm for managing platelet transfusion support throughout the course of a patient's treatment for leukemia.

Prevention of Alloimmunization

The contaminating lymphocytes in earlier preparations of platelet transfusions served as the major antigen-presenting component leading to alloimmunization to HLA antigens [159]. Animal studies document this, in that in murine studies, repeated administration of pure preparations of platelet suspensions was incapable of inducing an antibody response, but when the preparation also contained lymphocytes, an antibody response was induced [159]. Lymphocytes carry class II histocompatibility antigens that are necessary for invoking a recipient immune response against class I HLA antigens. Although class II antigens are not found on platelets, the presence of lymphocytes in the transfusion leads to activation of an immune response that is subsequently directed toward both lymphocytes and platelets (which carry class I antigens) and thus produces the development of alloimmunization and transfusion refractoriness [159, 160].

It appears that different levels of leukocyte depletion yield specific results. For example, a reduction to less than 10^7 leukocytes/transfusion will reduce the rate of transfusion reactions. However, it requires a reduction to less than 10^6 leukocytes to prevent transmission of cytomegalovirus (CMV) and to reduce the development of alloimmunization [92, 161, 162].

The TRAP study has demonstrated that this level of leukocyte depletion can be accomplished utilizing both modern apheresis techniques and modern filtration techniques, most with polyester, leukoadherent filters (all provide reduction of greater than 3 logs, to $<10^7$ leukocytes) [16, 28, 92, 161, 162]. These data are supported by other smaller studies that in fact provided the stimulus to engage in this randomized trial to test the hypothesis of leukodepletion and the prevention of alloimmunization [16, 28, 163–165]. In addition, in the TRAP study, a method of lymphocyte inactivation, utilizing ultraviolet B (UVB) irradiation, was administered to the transfusion products, to inactivate lymphocytes and antigen-presenting cells, such that they became incapable of generating an alloresponse [92, 166–168]. In animal studies, data

Table 54.6 Algorithm of platelet transfusion management

1. Preparation of a new leukemia or other patient requiring multiple transfusions
(a) Obtain HLA typing from patient and family and maintain record
(b) Transfusion history: prior pregnancies, transfusions, viral infections
2. Transfusion products
(a) RBCs: filtered or leukodepleted at blood center
(b) Platelets: Leukodepleted, random donor
• Pooled single units
• Apheresis
(c) Irradiated if potential transplant candidate or severely immunosuppressed
3. Reactions to platelets or poor 24-h increment
(a) Obtain 1 h or 10 min posttransfusion count
(b) Assess other clinical factors that may contribute to refractoriness
(c) Assess storage duration of platelets prior to transfusion
(d) Evaluate ABH compatibility
(e) Assess for presence of lymphocytotoxic antibodies
(f) Reaction, but adequate posttransfusion increment
• Initiate leuko-poor or filtered platelets if not done previously
• Continue to monitor early posttransfusion platelet counts
(g) Poor immediate posttransfusion increment
• Nonmatched but fresh single-donor product—get early posttransfusion count
• Family donor—possibly more compatible—get early posttransfusion count
• Consider HLA-matched platelet transfusion—get early posttransfusion count
• Consider cross-matched, ASP-selected platelets—get early posttransfusion count
4. Subsequent courses of treatment
(a) Reevaluate transfusion history to determine previous response to platelet transfusions
(b) Obtain lymphocytotoxic antibody (LCTAb) screening level
(c) If alloimmunized, refractory, or with a history of reactions or with high-titer LCTAb
• Initiate autologous platelet cryopreservation
• Be prepared to provide HLA-compatible or cross-matched platelet products
• Monitor all transfusions with early posttransfusion counts
(d) If no history of alloimmunization or refractoriness
• Begin with pooled single units or random single-donor apheresis platelets
• Monitor early posttransfusion counts
– May develop anamnestic antibody response
– Likely to continue to respond to random donor platelets

demonstrated the ability to prevent alloimmunization and to perhaps induce immune tolerance using this technique [166, 167]. Initial studies in humans demonstrated the functional integrity of platelets subjected to UVB irradiation as evaluated by posttransfusion platelet count increments [166, 168], although storage time was somewhat diminished [169]. Therefore, this technique was felt to be appropriate for study in the TRAP trial, since it was not known at the initiation of the trial whether the current techniques would produce sufficient leukodepletion to actually prevent alloimmunization.

Subsequently, the TRAP study has demonstrated that this level of leukodepletion or leukocyte inactivation by UVB does in fact lead to a major reduction in the rate of alloimmunization among patients with leukemia who are transfused multiple units of platelets and RBCs [92]. All three approaches (filtration, apheresis, and UVB inactivation) significantly reduced the rate of alloimmunization compared to unmanipulated platelet products, and they were all equally effective. Among the subjects who received nonmanipulated platelets, the rate of development of lymphocytotoxic antibodies was 45% (somewhat lower than that in many previous reports), but the rate of development of lymphocytotoxic antibodies among the other three treatment groups was 17–21%. The rate of alloimmunization leading to refractoriness to platelet transfusion was 13% among those who received standard, nonmanipulated, pooled random donor platelets (again, lower than that in many reports) compared to 3–4% in the patients in the other three groups [92].

The overall rate of refractoriness was 27%. Thus, the current platelet transfusion product recommended for patients expected to receive multiple platelet transfusions, such as those being treated for leukemia or expected to undergo SCT, is to provide leukocyte depleted platelets. Irradiated products are recommended for those who will undergo SCT and those with severe immunodeficiency, such as Hodgkin's disease. This will be discussed further subsequently.

Hazards of Platelet Transfusion

Platelet transfusions are usually very safe, and the benefits outweigh the potential risks. Nevertheless, certain hazards of platelet transfusion therapy should be considered. Transfusion reactions occur most commonly during or shortly after infusion (although they may be delayed if the patient is premedicated with acetaminophen) and are usually mild, consisting of a sensation of chilliness (rarely shaking chills) or fever, with or without urticaria. Urticaria is most often associated with plasma-related components. Reactions due to leukoagglutinins (white cell antigen–antibody reactions) are febrile and nonhemolytic. Leukocyte-poor platelet products have been shown to reduce the incidence of platelet transfusion reactions due to leukocyte-specific antigens and even to anti-HLA alloimmunization [170]. Some advocate removing plasma as well, as an immunologically active medium, containing reactive substances than may contribute to reactions [34, 171]. Stored platelets, if not prefiltered, may accumulate

cytokines released from leukocytes during the storage process, such as interleukin-6 (IL-6) among others. These may lead to a febrile reaction upon transfusion [33, 170–172]. The presence of IL-6 in the transfusion may also induce what appears to be an allergic reaction with urticaria [172].

Although with modern platelet preparation, red cell contamination is reduced, there is still considerable plasma, particularly with apheresis platelet products, which may contain antibodies to ABH blood types. Therefore, ABH-mismatched platelet transfusion may rarely be associated with a hemolytic reaction if the donor has a high-titer hemagglutinin [173–176]. These have been reported over the years as case reports, but Larsson et al. suggests that this type of reaction may be underrecognized in a population receiving numerous blood products [174]. Additionally there has been a greater utilization of apheresis platelets with considerable plasma volume. Fontaine et al. implemented a risk mitigation protocol which includes volume reduction of ABH incompatible platelet units [175].

Circulatory congestion is a common, preventable problem in very ill patients receiving multiple infusions. This develops not only from the individual transfusions being administered, but also from the additive effects of administration of all blood products, multiple antibiotics, and other intravenous medications. If necessary, the volume of plasma in a platelet transfusion can be decreased immediately before infusion, using centrifugation and resuspension in a smaller volume of plasma. This is routinely done for transfusions to children.

Transfusion-related acute lung injury (TRALI) may be an unrecognized cause of non-cardiogenic pulmonary edema following platelet transfusion. This is usually a reaction from administration of anti-neutrophil and/or anti-HLA antibodies in the plasma of platelet units, and possibly other bioactive substances into susceptible recipients. In the past decade, TRALI-risk reduction policies for donor screening have been implemented in a number of countries and have been widely accepted [177, 178]. Essentially, female platelet donors with history of prior pregnancies trigger HLA-Ab testing and those who are positive are redirected to whole blood donation. There has been a dramatic reduction of TRALI by more than two-thirds in both the United States and United Kingdom with this approach, with fatalities similarly greatly reduced [178].

Bacterial contamination of platelets is rare, but it is a documented cause of platelet transfusion reaction from stored platelets. This must be considered in severe reactions, and the transfusion bag and patient should be cultured [179]. Because platelets are currently stored at room temperature, the risk of bacterial growth exists with storage [180, 181]. New methods of testing for bacterial contamination are being applied pre-transfusion [182, 183]. Trials of pathogen reduction storage systems are underway worldwide, including the use of UV irradiation with or without additives [64–66]. As noted in the section on platelet storage, 7-day storage has recently been approved when used with the Verax Platelet PGD test as a pre-infusion test for pathogen screening [47, 48]. Additionally a pathogen reduction system called Intercept has been approved for use in the United States for stored apheresis platelets which utilizes amotosalen and UVA illumination to irreversibly cross-line nucleic acids in potential pathogens [184]. A system called Theraflex UV is being studied in Australia for pathogen control of emerging viral infections [185].

Clearly, a major concern in transfusion support is the transmission of infectious diseases carried by donors. Serologic testing of donors for hepatitis viruses has markedly diminished transfusion-related transmission of hepatitis [182, 183]. As noted above, several of the systems undergoing investigation are directed at emerging, and often subclinical infections among donors [184, 185].

Leukocyte-associated viruses may also be transmitted by transfusion, and these include human T-cell leukemia virus types 1 and 2 (HTLV-1 and 2), CMV, and the Epstein–Barr virus (EBV). Systematic donor screening is routinely performed for HTLV-1 and -2 to eliminate donors carrying these viruses. It has also been shown that the incidence of CMV infections developing in CMV-negative recipients as a result of transfusion can be modulated by limiting the number of CMV-positive donors and by adding CMV-immune globulin [186]. However, the ubiquitous nature of the CMV carrier state makes it nearly impossible to completely supply blood product support to very ill patients with strictly CMV-negative donors. The use of leukocyte-depleted blood products also markedly reduces exposure to CMV by reducing the number of virus-carrying leukocytes and in many cases prevents the development of primary CMV infection in a CMV-negative recipient [186–190]. The AABB recently published a Committee Report on reducing transfusion-transmitted CMV, and this describes the variation in practice to control this entity [191].

Another recognized complication of transfusion that is receiving increased attention is transfusion-associated graft-vs.-host disease (TA-GvHD). This phenomenon is mediated by immunocompetent donor lymphocytes that activate against the foreign recipient. Although this has been a long-standing problem in bone marrow transplantation, this has also been described in the nontransplant setting and in infants [192]. In addition, immunocompetent recipients undergoing whole blood transfusion from haplotype-identical donors have developed this syndrome [193–195]. Because of the haplo-identity of the donor, usually a relative, the recipient recognizes the donor as self, but the donor cells react against the recipient's nonidentical haplotype.

The clinical syndrome is usually severe, with erythematous rash, fever, diarrhea, and abnormal liver function tests.

A skin biopsy is often diagnostic, with infiltration of lymphocytes along the dermal–epidermal junction, basal cell vacuolar degeneration, hyperkeratosis of the stratum corneum, and degenerative dyskeratotic cells that may be accompanied by lymphocytes, known as satellite dyskeratosis [195–198]. HLA typing of the donor and patient may also give an indication of the cause of the syndrome. TA-GvHD is often rapidly progressive and frequently fatal.

Patients with hematologic malignancies, particularly those undergoing intensive treatment, are considered to be at increased risk for TA-GvHD, although several studies have demonstrated that the reported incidence in the USA is much lower than would be expected by analysis and prediction from HLA-type frequencies [197, 198]. Clinical reports in the USA are also relatively rare considering the frequent use of family donors. Studies from Japan implicate the use of fresh whole blood and a homogeneous population in the development of this phenomenon [199]. Other studies have suggested that the degree of T-cell competence or T-cell depletion of the recipient, such as in Hodgkin's disease and perhaps non-Hodgkin's lymphoma, is important in the manifestation of this syndrome [196, 197]. Current standards recommend that blood products from directed donors that are first or second degree and to recipients who are significantly immunoincompetent be irradiated, such as transfusion to those with Hodgkin's disease [23, 196, 197]. Currently, most patients with hematologic malignancies who are in consideration of transplant or who are immunoincompetent are receiving irradiated blood products. Studies are under way to evaluate the effect of gamma irradiation and ultraviolet irradiation on both platelet function and leukocyte inactivation.

Granulocyte Transfusion Therapy

The concept of utilizing granulocyte transfusions for treatment of neutropenic sepsis arose out of the successful use of platelet transfusions in controlling bleeding. As a result of that success, patients undergoing treatment for leukemia were at much lower risk of mortality from severe bleeding; however, they frequently succumbed to infection. The development of technology capable of separating granulocytes from whole blood led to the evaluation of granulocyte transfusions in severely infected neutropenic patients. The ongoing limitation of this cellular support continues to be the ability to provide sufficient numbers of granulocytes to eradicate infection in severely infected, severely neutropenic patients. Providing donors that are ABO matched, at a minimum, on a daily basis, who are accepting of premedication including dexamethasone and/or granulocyte colony stimulating factor (G-CSF) all add to the complexity of supporting such a severely ill patient through what is expected to be a temporary situation. Nevertheless, the experience of many investigators and clinicians has shown efficacy in many cases, and this continues to stimulate research and innovation in providing this critical supportive cell therapy.

The initial investigations utilized granulocytes from patients with chronic myelogenous leukemia (CML), to take advantage of the elevated WBC count, which allowed collection of large numbers of granulocytes. This produced both an elevated posttransfusion WBC count in the recipient, and a clinical benefit [3, 200, 201]. Evaluation of the function of these cells showed responsiveness to bacterial infections [202]. These transfusions successfully eradicated infection, including gram-negative organisms [3, 200, 201]. Collections of granulocytes from patients with CML were in fact the first "peripheral blood stem cell transfusions." They included not only mature neutrophils, but myeloid precursors, primarily committed, leading to temporary engraftment with persistent production of myeloid cells and sustained neutrophil counts of more than 500/μL for 4 or more days after the transfusion [3, 201]. These initial investigations pointed out the principles necessary for success of granulocyte transfusion therapy: a sufficient dose (more than 10^{10} neutrophils) and the sustained presence of infection-fighting cells.

Despite the success of these early investigations, the use of CML donors was not practical in terms of donor availability as well as new, more effective treatments for patients with CML. However, it provided a proof of principle that with sufficient dose and duration, granulocyte transfusions could aid in eradicating severe infection in neutropenic patients. Therefore, granulocyte transfusion research has focused on optimizing granulocyte collection from normal donors, with improvements in apheresis technology and strategies to prestimulate donors to enhance collection numbers.

Granulocyte Collection and Storage

Granulocyte collection technology has followed steps similar to those of platelet collection and utilizes the same principles of differential centrifugation to obtain the selected blood component. Modern techniques utilize the apheresis technology described in Chap. 52, with the best yields obtained with continuous-flow leukapheresis. Granulocytes are difficult to separate from whole blood because they are essentially the same density as RBCs. Therefore, whether apheresis or gravity leukapheresis is utilized, additional techniques are necessary to induce the efficient separation of granulocytes from RBCs [6, 203, 204].

This involves the use of sedimenting agents that induce the formation of erythrocyte rouleaux so that "clumps" of RBCs become heavier than granulocytes and the two cell types can be centrifuged apart. Hydroxyethyl starch (HES) and low molecular weight dextran have been used for this purpose [205]. Both agents coat the red cell surface, induce

the formation of rouleaux, and allow efficient differential centrifugation, with sedimentation of the red cell clumps. Since granulocyte transfusions are collected from normal donors, the effect of starch exposure in donors has been evaluated. At 24 h, 50% of the HES remains in the blood of the donor, and traces of the substance have been found as long as 8–9 months after a leukapheresis procedure [206]. No long-term effects have ever been reported in either donors or recipients in more than 30 years of leuapheresis utilizing this substance. HES is also widely used as a volume expander in surgical and intensive care patients with no long-term ill effects reported.

Granulocytes, particularly for adult recipients, are almost exclusively collected using apheresis machines (see Chap. 52 for details) [6, 204]. Techniques of granulocyte collection of historical interest include a manual technique called gravity leukapheresis, in which 1 U of whole blood was removed and sedimented with HES; the granulocytes were then separated, and the red cells were reinfused. This cycle was repeated until an adequate number of granulocytes were collected, similar to the gravity method for platelet collection [5, 203]. Reports from Japan have utilized a “simple bag method” to obtain granulocytes from growth factor-stimulated donors without apheresis for pediatric recipients [207].

The major technical problem limiting granulocyte transfusion efficacy has been the inability to obtain a sufficient dose of granulocytes from normal donors to be effective in combating sepsis in neutropenic patients. Evolution of granulocyte collection from gravity methods to continuous flow apheresis improved yields [208, 209], as did the adoption of premedication with corticosteroids (dexamethasone 8 mg orally 12 h prior to the procedure, and 10 mg as a slow intravenous infusion immediately preceding the procedure) to induce marginalized granulocytes to enter the blood stream [210]. Even with all of these improvements, however, the yield of granulocytes ranged from 2.5 to 5×10^{10} granulocytes for each donation [208–210]. This is still 1/100th of the estimated intrinsic response to sepsis. Functional studies demonstrated normal antibacterial function of granulocytes collected using steroid premedication and HES sedimentation [211].

Several reports have demonstrated that premedicating normal donors with G-CSF with or without dexamethasone further enhanced the yield of granulocytes [212, 213]. Donors receive single or multiple doses of G-CSF and donor WBC counts reach as high as 50,000/μL, with mean granulocyte collection yields of 4×10^{10} (range, $1–14 \times 10^{10}$) [212]. Recipients in these initial studies demonstrated sustained circulation of transfused granulocytes and no significant ill effects were noted in the donors [212, 213]. Granulocyte function studies in these collections showed normal chemotaxis and phagocytosis, and possibly enhanced enzymatic activity, demonstrated by chemiluminescence and superoxide formation [212, 214–216]. Reports in pediatric oncology, where there is a high granulocyte dose to body surface area (BSA) ratio, suggest clinical benefit with increasing doses of cells per BSA [207, 217, 218].

Studies have evaluated the efficacy and tolerability of G-CSF alone, dexamethasone alone, or the combination, with reductions in doses of G-CSF and individual centers have their preferred granulocyte mobilization regimen [219–224]. The ability to premedicate donors and provide daily or every other day granulocytes is most efficiently achieved within the setting of a cancer center with apheresis capability and where patient—related donors are often highly motivated to provide products for a specific patient. Nevertheless, Price et al. have demonstrated the ability to provide maximally stimulated granulocytes in high yields utilizing community donors who are also highly motivated [225].

The ability to collect larger and potentially more therapeutically useful doses of granulocytes has stimulated interest in revisiting the issue of granulocyte storage. Historically, even short-term liquid storage of granulocytes has been extremely difficult due to the high metabolic activity of these cells. The number of morphologically normal granulocytes in standard storage conditions declines by 10–15% per day during the first 2 days of storage. Studies have shown rapid exhaustion of glucose in the storage media [226], with measurable decreases in functional parameters such as microbial killing, chemotaxis, and the ability to circulate within the first 24 h of storage [227, 228]. Alterations in expression of adhesion molecules have also been described, which may contribute to these functional deficiencies [229]. One study evaluated the storage potential of granulocytes mobilized with G-CSF [230]. Although the hypothesis was that these cells might be less metabolically active, in fact the pH was less than 7.4 at the time of collection, and was less than 6.0 after 24 h of storage. To maintain storage pH, mobilized granulocyte concentrates required dilutions by 1–8 or 1–16, neither of which is practical [230].

Other investigations of enhancing granulocyte storage have evaluated the addition of G-CSF to the storage medium [216, 231, 232], and some investigators have reported successful 24-h storage with this approach in conjunction with reduced temperature [232]. Further evaluation is needed, and no standard long-term storage practice currently exists.

Thus, current practice dictates that granulocytes be transfused as soon after collection as possible. Donors must be pretested so that the product can be transfused within hours of collection. Even for short-term storage (meaning hours), granulocytes should be placed in large-volume bags (1000–2000 mL) with a large volume of plasma to avoid loss of function. Storage should, however, be avoided if at all possible.

Criteria for and Evaluation of Normal Donor Granulocyte Transfusions

Although the use of granulocyte transfusions has diminished considerably since the late 1970s, the clinical indications remain the same [233–235] (Table 54.7). Granulocyte transfusions should be considered in patients who are severely neutropenic (neutrophil counts less than 500/μL), whose marrow is not expected to recover imminently but will eventually, and who have a severe infection such as pneumonia or septicemia that is not responding to appropriate antibiotics.

Fortunately, this clinical setting occurs less frequently than previously, at least for bacterial infections, due to more knowledgeable use of empirical antibiotic approaches, and better and more broad-spectrum antibiotics. The latter can lead to resistance, however, and therefore may yield another scenario in which granulocytes may be considered. Fungal infections remain a difficult therapeutic dilemma in granulocytopenic patients. Therefore, infections remain the major cause of morbidity and mortality in neutropenic patients. Mobilization of donor granulocytes with growth factor stimulation in addition to dexamethasone prior to collection has yielded larger numbers of granulocytes, which it is hoped will lead to improved efficacy, particularly in patients with sepsis or fungal infections [234–236].

Studies evaluating granulocyte transfusions in patients with fungal infection must be attentive to dose of granulocytes, and such transfusions must be considered early in the clinical course, preferably prior to positive blood cultures. Doses less than or equal to 1×10^{10} are unlikely to be particularly effective, as noted from prior studies with cells from CML patients and prior animal studies [3, 236–241]. Data from studies in animals and with CML donors suggest that more than 10^{10} neutrophils in a 70-kg person is the minimum requirement for efficacy (1.5×10^8/kg) [239–241], which is one-tenth of the daily production of neutrophils in a normal, noninfected adult. Two studies reported yields of $2–4 \times 10^{10}$ neutrophils/transfusion with growth factor-stimulated donors, and some investigators have recommended that amount as the new standard for granulocyte transfusion therapy [212, 242]. Evaluation of the potential clinical value of granulocyte transfusions in infected, neutropenic patients will require an optimal dose and duration of granulocyte transfusion therapy. Thus, when technical factors do not limit the modality, it can best be evaluated, in the context of the data from the older CML donor transfusions.

Table 54.7 Criteria for initiating granulocyte transfusions

Criteria
Severe neutropenia (neutrophils <500/μL)
Marrow shows no evidence of immediate recovery
Clinically significant infection such as pneumonia or septicemia
Failure or inadequate response to appropriate antibiotics
Fungal infection poorly responsive to appropriate antibiotics
Expectation of eventual marrow recovery

Contemporary single institution reports, utilizing growth factor stimulated donors, both retrospective and prospective, again report major clinical benefit in a proportion of patients [243–247]. The variable outcomes reflect the impact of clinical factors, donor availability, and technical issues. A Cochrane analysis of the few randomized trials including old studies of lower doses and even prophylactic transfusions describes data quality issues related to enrollment biases, technological issues, and granulocyte doses [248, 249]. Because granulocyte transfusions, even in studies, are given in clinically emergent situations, the ability to control variables is limited.

A recent report of a prospective, randomized trial, RING—Resolving Infection in Neutropenia and Granulocytes, demonstrates the complexities and difficulties of controlling variables and conducting studies in this modality [250, 251]. The trial randomized infected patients, using specific criteria, to either standard antimicrobial treatment or the addition of granulocytes, mobilized by G-CSF/dexamethasone. The goal was to evaluate the efficacy of transfusion of high doses of granulocytes in seriously infected patients. Even in the setting of this trial, the results cannot be interpreted due to major limitations: (a) only half the planned number of patients were accrued (114 of the planned 236 subjects, with 56 receiving granulocytes)—interpreted as patient/clinician sense of lack of equipoise, (b) patients eligible might not have been routinely selected for granulocytes, and (c) major dose issues, with more than 25% receiving less than the prespecified dose of granulocytes, some substantially less according to the report [250]. Of importance is that when a subset analysis was conducted, based on dose of granulocytes administered, there indeed was a statistically significant benefit to those who received the high dose of granulocytes [250]. Therefore, the ability to maximize dose of granulocytes, even with pretreatment mobilization, remains a technical challenge. Nevertheless, with optimal circumstances, as reported in numerous small studies, granulocyte transfusion appear to provide clinical benefit. Optimizing this approach will require further research.

A commentary in reference to this paper suggests that perhaps mature granulocytes are not the best source of cellular anti-infection therapy, given their short life-span after collection (10 h), the technical issues of pre-screening and premedicating donors, the continued issues with obtaining sufficient doses of granulocytes, and the emergent requirement of providing a product within 6 h of collection [251]. This concept is leading to new avenues of research (see future directions).

Histocompatibility Issues in Granulocyte Transfusions

Febrile reactions and subsequent pulmonary infiltrates have been observed following granulocyte transfusions [249]. Although not always detected after such reactions, alloimmunization is a potential cause of reactions and has been documented in several studies [252, 253]. Granulocytes have both HLA antigens and neutrophil-specific antigens (the latter not linked to HLA) on their surface.

Observations from early granulocyte transfusion studies suggested a rough correlation between the occurrence of transfusion reactions and recipient alloimmunization, with the presence of antibody directed against the donor leukocytes [254]. Clinical studies suggested that alloimmunization to platelet transfusion (antibodies to HLA antigens) also affected the clinical outcome of granulocyte transfusions [255–257]. Studies imaging the infusion of Indium-111-labeled granulocytes showed more definitively that documented alloimmunization prevented the migration of granulocytes to sites of infection [258, 259]. Studies of Indium-111-labeled granulocytes in alloimmunized patients also demonstrated prolonged uptake in the lungs, pointing to alloimmunization as a component of the pulmonary reactions sometimes noted with unmatched granulocyte transfusions [260, 261]. One study evaluated granulocytes obtained from colony-stimulating factor-mobilized granulocytes and also described an effect of leukocyte compatibility, evaluating lymphocytotoxicity as a major factor in granulocyte increment and survival after transfusion [262]. Another study of granulocyte transfusions given to patients with severe aplastic anemia and serious infections did not observe a difference in increment despite HLA antibody production [263].

Granulocyte donors are currently selected based on ABH compatibility. However, testing for leukocyte antibodies is variably performed [261, 262]. If no evidence of clinical alloimmunization exists, then ABH compatible granulocyte transfusions are given. In patients who are already alloimmunized to platelet transfusions, proceeding with granulocyte transfusions can be hazardous, as described earlier. To proceed, donors who are effective platelet donors for alloimmunized patients are the safest candidates for granulocyte donation. No standard compatibility approach has yet been established, in part due to the rarity of the use of this transfusion product and its often urgent request. In the RING trial, volunteer donors were not chosen by HLA or granulocyte compatibility [250].

Reactions and Risks of Granulocyte Transfusions

All of the commonly reported types of blood product reactions can occur with granulocyte transfusions. Nonspecific and possibly multifactorial reactions include fever, urticaria, and fluid overload. Other potential causes of severe pulmonary reactions include the appropriate migration of the neutrophils to the site of the pulmonary infection with or without the development of an infiltrate on a radiograph and, more seriously, acute pulmonary edema and adult respiratory distress syndrome. The latter may be a result of an immunologic reaction (i.e., alloimmunization) or infection and the release of inflammatory cytokines. Pulmonary decompensation has also been associated with the combined administration of granulocyte transfusions and amphotericin B in one series [264], but less definitively causal in several other reports [265–267]. Adverse reactions including pulmonary reactions have been documented with either granulocytes or amphotericin B alone so that either or both may be the causative factors in reactions when they are administered concurrently. However, although the risk of reactions appears to relate to each individual component, prudence dictates that whenever possible, granulocyte transfusions and amphotericin B be administered separately, and with several hours between administrations [266]. In the contemporary RING study, transfusion reactions within 6 h of granulocyte transfusion were recorded, and included grade 1–2 (mild/moderate) in 41% of recipients (fever, chills, mild change in blood pressure). More severe reactions (grade 3–4) occurred in 10 patients (20%), including hypoxia ($n = 7$), tachycardia (n − 1), hypotension ($n = 1$), and allergic reaction ($n = 1$). One patient had grade 4 hypoxia requiring temporary ventilator support [250]. As noted above, donors were matched by ABH only.

Again, as with other blood products, the potential for TA-GvHD exists, particularly since family donors are often utilized for granulocyte transfusions. The AABB has recommended irradiation of all cellular components donated by relatives. The potential effect on viability adds emphasis to the recommendation that granulocytes be transfused as soon as possible after collection to ensure a viable and effective blood product.

Finally, the transfusion of granulocytes increases the risk of transfusing cells containing CMV. Both donors and recipients should be screened prior to initiating granulocyte transfusions. Although a large majority of the population has already been exposed to CMV with antibody titers, and therefore do not require CMV-negative blood products, the occasional recipient may be negative and will therefore require CMV-negative granulocytes. If the clinical infection is so severe that granulocytes are a necessity, then weighing the risk/benefit ratio of CMV-positive products becomes a very difficult decision. An acute CMV infection due to transfusion is less likely to occur during standard induction therapy for leukemia, but could potentially be fatal in the allogeneic transplant setting.

Future Potential for Granulocyte Transfusion Therapy

Granulocyte transfusions are infrequently used today, even though infection is still the major cause of death in neutropenic leukemia and bone marrow transplant patients.

A resurgence of interest has occurred due to the ability to provide transfusions with enhanced numbers of granulocytes, and due to the frequency of either resistant bacteria or fungal infections [243–251]. Several investigators have commented that granulocyte transfusion was prematurely abandoned, and we must now relearn their use with safer, more effective technology for collection [233, 251].

Additional research is ongoing to develop related cellular products such as myeloid progenitor cells with the capability of differentiating and providing an ongoing but temporary source of granulocytes [268, 269]. Animal studies have demonstrated the ability to recover from lethal challenges of bacteria and fungus using co-transplantation of lineage-specific progenitors [268]. A phase I trial in neutropenic leukemia patients has been reported and a phase II trial is ongoing [270, NCT 02282215]. Other studies are exploring an approach to generate functionally mature granulocytes induced from pluripotent stem cells [271, 272]. The potential for granulocyte support clearly demonstrates a need, and innovative approaches are undergoing investigation to supply this need.

Conclusions

The development of successful transfusion supportive care has had a major impact on our ability to treat patients with hematologic malignancies and those undergoing bone marrow transplantation. Additionally, transfusion immunology has contributed to the overall understanding of allogeneic transplant immunology. We continue to advance technology to make blood component therapy and transfusion a safer modality. Both platelet and granulocyte transfusion will remain a part of our armamentarium in the treatment of hematologic malignancies, and further optimization of these lifesaving components will continue. They are critical to the current management of these patients.

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55

Alternative Sources of Hematopoietic Stem Cells and Their Clinical Applications

Filippo Milano, Shelly Heimfeld, and H. Joachim Deeg

History

Reports on the therapeutic use of bone marrow for the treatment of anemia associated with leukemia or parasitic infections date back about one century (reviewed in [1]). However, it was not until the events at Alamogordo, New Mexico, and the observations in atomic bomb casualties at Hiroshima and Nagasaki that systematic research into hematopoietic stem cell transplantation (HSCT) got under way [2]. Studies on total body irradiation (TBI) in mice, rats, dogs, and nonhuman primates revealed three levels of TBI-related injury: a marrow (hematopoietic) syndrome at about 500–700 cGy, an intestinal syndrome at 1200–10,000 cGy, and a cerebral syndrome at even higher doses [3]. In mice, shielding of the spleen during TBI, implantation of a syngeneic spleen, or infusion of syngeneic spleen or marrow cells after TBI rescued animals from the marrow syndrome [4, 5]. Intravenous injection was the most effective way of transplanting hematopoietic cells, which "homed" to the marrow cavity and other hematopoietic organs. These studies also showed that hematopoietic stem cells (HSC) were present at sites other than the marrow and were viable in the circulation. While the infusion of autologous or syngeneic cells rescued animals without complications, animals given cells from allogeneic donors developed a "secondary disease," subsequently known as graft-vs.-host disease (GVHD) [3, 6].

The first modern clinical transplant attempts were undertaken in patients with end-stage leukemia beginning in 1957. Results were discouraging: patients transplanted from syngeneic twin donors had a smooth posttransplant course, but generally died from progressive leukemia. Patients transplanted from allogeneic donors, on the other hand, generally died with severe GVHD [7]. The development of GVHD was recognized to be due to histoincompatibility between donor and recipient first described by Gorer in mice [8] and then characterized in humans by Dausset and others [9, 10].

Characterization of Hematopoietic Stem Cells and Sites of Hematopoiesis

A stem cell is defined by the ability to generate progeny while maintaining this capacity by way of self-renewal. Hematopoiesis is a continuous developmental process in which stem cells make specific cell fate decisions to produce various blood cell lineages [11]. The consistent generation of appropriate numbers and types of mature cells, as well as the maintenance of the HSC pool, requires a regulatory network, which is progressively being characterized. Within the hematopoietic microenvironment a complex signaling network involving soluble and cell-bound cytokines, as well as interactions among hematopoietic cells and stromal elements, has evolved to appropriately regulate the differentiation and proliferation of HSC and progenitor cell populations [12]. Despite considerable experimental and laboratory progress, the molecular signals that mediate cell fate specification and the self-renewal of HSC remain incompletely understood and controversial [11–14]. Recent studies using refined phenotypic and genetic analysis have generated data that challenge the established model of precursor cells leading to common myeloid/lymphoid and erythroid/megakaryocytic branches [15, 16].

Hematopoiesis in vertebrates evolves through three embryological stages [17]: the mesoderm is formed near the primitive streak, and mesodermal cells begin to migrate along the amniotic wall and visceral endoderm to form the first visceral blood islands (mesodermal or yolk sac phase). These islands then become connected by blood vessels of the primitive circulatory system. Later in embryogenesis, the

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spleen and liver become the primary sites of hematopoiesis (hepatosplenic phase) before eventually the bone marrow takes over (medullary phase). At the time of birth, the marrow has become the predominant site of hematopoiesis in humans; only under stress conditions—such as in disease states—will hematopoiesis occur in the spleen, liver, and other extramedullary sites during adult life. In other species, e.g., mice, the spleen remains a hematopoietic organ throughout life.

In 1951, Brecher and Cronkite showed that cross-circulation between normal rats and rats irradiated with marrow-ablative doses of TBI rescued the irradiated animals by way of engraftment of hematopoietic cells originating from the nonirradiated partner [18]. Similar results were obtained subsequently in mice, baboons, dogs, sheep, and other species, showing that precursor cells capable of long-term hematopoietic reconstitution were present in the circulation. HSC are also present at relatively high concentrations in umbilical cord blood (UCB) [19], an extracorporeal segment of the fetal circulation.

Studies with candidate human HSC have essentially been confined to in vitro or xenogeneic animal model assays since it is ethically unacceptable to experimentally test various cell populations in humans for their long-term repopulating ability. In vitro characterization has involved Dexter-type long-term cultures, blast cell assays, assays for long-term culture initiating cells, or high proliferative potential colony-forming cells (reviewed in [20]). Early progenitors have also been identified phenotypically [21], and have been transplanted and characterized in immunodeficient (SCID) or SCID/NOD (non-obese diabetic) mice as well as in NSG (NOD/SCID/gamma) mice or sheep and in some clinical trials [22, 23]. According to the most prevalent opinion, the earliest identifiable hematopoietic precursor in human adult marrow is $CD34^{+}$ $CD38^{-}$ $CD33^{-}$ $HLA\text{-}DR^{-}$ $Thy1^{lo}$ $c\text{-}kit^{lo}$ ($Rho123^{dim}$) and noncycling [24]. However, this view has been challenged, and recent data suggest the existence of $CD34^{-}$ precursors that are more primitive than $CD34^{+}$ cells [25, 26]. $CD34^{+}$ cells account for 1–2% of nucleated marrow cells, and about 1% of those are $CD38^{-}$ DR^{-}. In peripheral blood of healthy individuals cells with similar characteristics are present, albeit at 100-fold lower concentrations [27]. Primitive stem cells in umbilical cord blood appear to be DR^{+} [28]. HSC from all these sources are further characterized by the lack of expression of lineage-specific markers.

Rationale for Hematopoietic Stem Cell Transplantation

Depending on the disease being treated and the patient's characteristics, the rationale for HSCT, and consequently donor selection and preparative regimen, vary. HSCT is widely used to overcome dose-limiting hematopoietic toxicity of chemoradiotherapy, to mediate disease elimination via an allogeneic graft-vs.-tumor (GVT) effect, and to replace congenitally defective hematopoietic systems or repair congenital metabolic or immune disorders. Encouraging results are also obtained in the treatment of autoimmune diseases. Thus, congenital and acquired, malignant and nonmalignant diseases are being treated successfully with HSCT. The use of HSC as "vehicles" for gene therapy remains investigational. Lymphohematopoietic cells have also been used in conjunction with solid organ transplantation to induce tolerance [29, 30].

Dependent upon the indication, HSC may be collected from the patients themselves (autologous) or from donors other than the patient (allogeneic), or, in rare cases, from an identical twin (syngeneic).

Autologous HSCT is utilized primarily as treatment of several malignancies in which dose intensification of therapy to myeloablative levels is potentially curative or capable of delaying disease recurrence (see below). For patients undergoing autologous HSCT to treat autoimmune disorders, the rationale is that intensive conditioning eradicates autoimmune cells and may allow a new and healthy repertoire of immune cells to regenerate and selectively increase regulatory T cells [31]. Active investigation of gene therapy to treat primary immune deficiencies, hemoglobinopathies, and metabolic diseases is ongoing. Allogeneic HSCT, in addition to hematopoietic reconstitution, contributes a GVT effect with curative potential for various malignancies [32]. This effect, mediated by donor lymphocytes, results in destruction of residual disease cells in the patient. Recognition of the GVT effect has led investigators to develop minimally toxic yet adequately immunosuppressive preparative regimens that allow for engraftment of donor cells and development of the GVT effect. These regimens (see next section) can be administered even to older individuals or patients with comorbid conditions who cannot tolerate the toxicity that may be associated with high-intensity conditioning regimens. However, because a GVT effect may take time to develop and is unlikely to have the capacity to overcome a high disease burden, these regimens are most effective in patients with indolent diseases or in patients in complete remission (CR) at the time of transplantation [33–35]. For younger, fitter patients, and patients at higher risk of relapse, high-intensity conditioning is generally still desirable prior to allogeneic HSCT to minimize disease burden and decrease relapse risk [36].

Allogeneic HSCT is also used as a curative strategy for nonmalignant congenital or acquired conditions that result in ineffective hematopoiesis, immunodeficiencies, or certain metabolic disorders. The allograft corrects the

deficiency and reconstitutes a normal hematopoietic system. Here, the goal is to minimize regimen-related toxicity and avoid GVHD. Because these patients often have not been treated with cytotoxic, immunosuppressive therapies prior to HSCT, their immune systems are frequently more intact, and securing engraftment can be more challenging [37, 38].

Preparation for Transplantation

High-Intensity (Myeloablative) Regimens

These intensive regimens ablate the patient's marrow and if not salvaged with HSC infusion would result in aplasia or severely prolonged cytopenias. High-intensity preparative regimens generally include chemotherapeutic agents such as busulfan, etoposide, melphalan, thiotepa, cytarabine, carmustine, and are often combined with irradiation, either in the form of TBI, limited field irradiation, or targeted radioimmunotherapy.

Reduced-Intensity Conditioning (RIC) Regimens

These regimens are designed to be less toxic than high-intensity regimens. However, the separation of conditioning into two intensity groups is somewhat artificial as there is a broad spectrum. The least intense and minimally toxic regimens provide immunosuppression just sufficient to allow engraftment of donor cells. Mixed chimerism of donor and host cells frequently develops for a period following transplantation. For nonmalignant disorders, mixed chimerism may be sufficient to correct the underlying condition, but to treat malignancies, complete donor chimerism is generally desired as the persistence of host cells tends to be associated with a high probability of relapse.

Two gray (Gy) TBI ± 90 mg/m^2 of fludarabine is the paradigmatic low-intensity regimen for patients undergoing HSCT for malignant diseases [39]. However, for patients undergoing transplantation for nonmalignant conditions, or those who have not received significant prior therapy for their malignancies, additional immunosuppression may be required to secure sustained engraftment. Modest increases in radiation doses, or added T-cell-specific therapies such as antithymocyte globulin (ATG) or alemtuzumab (anti-CD52 antibody), may be incorporated into these regimens [40, 41].

Fludarabine combined with modest doses of busulfan, cyclophosphamide, melphalan, or TBI are frequently used, somewhat "intensified" regimens [42–45]. Treosulfan, a busulfan analogue with less liver toxicity and possibly greater antileukemic potential, is currently under investigation as a novel agent in various regimens [46, 47].

Radioimmunotherapy has also emerged as a strategy to intensify radiation therapy with minimal toxicity. Radiolabeled antibodies targeting receptors expressed on hematopoietic elements allow for high dose internal radiation exposure of disease tissue/cells with minimal exposure of other organs. Radiolabeled CD20 antibodies are well established in the treatment of lymphoma, and radiolabeled CD45 antibody is effective in the treatment of myeloid leukemia and myelodysplastic syndrome (MDS) [46, 48]. New strategies to pretarget radiolabeled antibodies directly to malignancy may lead to even greater specificity and efficacy of this strategy [49].

Selection of Hematopoietic Stem Cell Donors

Three major questions are addressed when deciding upon the HSC source. First, will the transplant be autologous or allogeneic? Second, for allogeneic transplantation, will the donor be related or unrelated? Third, will the stem cells be collected from the bone marrow or peripheral blood or will cord blood be used? Characteristics of these stem cell sources are summarized in Tables 55.1 and 55.2.

Table 55.1 Hematopoietic stem cell sources

Source	Indication
Marrow/peripheral blood	
HSC in peripheral blood originate from marrow and recirculate to the marrow. Their presence in peripheral blood makes them readily accessible. However, their differentiation status may differ from that of marrow HSC, and they are further modified by exposure to G-CSF, which "mobilizes" them from the marrow. PBSC lead to faster hematopoietic recovery posttransplant than cells aspirated from unmanipulated marrow. Also, PBSC can possibly be obtained even if aspiration from marrow is not possible.	
Allogeneic[a]	Acquired diseases with or without HSC involvement; congenital disorders
Syngeneic	Acquired malignant or nonmalignant diseases with or without HSC involvement
Autologous	Acquired, usually malignant disorders, generally without HSC involvement; autoimmune disorders; possibility of genetic correction of defect
Umbilical cord blood	
Allogeneic[a]	Lack of alternative stem cell source; acquired diseases with or without HSC involvement; congenital disorders
Autologous	Experimental; possibility of genetic correction of defect

[a]Includes related and unrelated donors

HSC hematopoietic stem cells, *G-CSF* granulocyte colony-stimulating factor, *PBSC* peripheral blood stem cells

Table 55.2 Characteristics of marrow, peripheral blood, and cord blood used for allogeneic HSCT

Characteristic	Marrow[b]	Peripheral blood	Cord blood
Harvest procedure	Anesthesia—Multiple marrow aspirates	Vascular access—Apheresis (≈4 h × 1–2)[a]	Extraction of cells from cord and placenta
Number of cells obtained (per kg patient weight)			
• Total mononuclear	$1–4 \times 10^8$	$2–100 \times 10^8$	$0.7–30 \times 10^7$
• CD34+	$1–5 \times 10^6$	$2–20 \times 10^6$	$<1–45 \times 10^5$
• T cells	$5–50 \times 10^6$	$2–10 \times 10^8$	$<5–>20 \times 10^6$
Cell subpopulations			
• CD34+		↑ #	↑ #
• T cells		↑ #; ↓ Reactivity	↓ Reactivity
• Monocytes		Increased IL-10	Reduced HLA-DR, CD86, ICAM1, IFN-γ production

[a]G-CSF mobilized
[b]Some results with marrow cells following G-CSF administration have been reported recently [50, 51]

Autologous vs. Allogeneic Transplantation

As described earlier, autologous transplantation is indicated for several diseases. While autologous HSC in principle should be available for every patient (except possibly heavily pretreated patients or patients with marrow failure states who are unable to mobilize sufficient stem cells for subsequent transplantation), autologous transplantation conveys no GVT effect, and there is always concern that some malignant cells are returned to the patient with the transplant inoculum. Until more effective gene transfer strategies are developed, autologous HSC are also not useful for genetically determined disorders. Given its inherent risks, allogeneic transplantation is generally reserved for diseases not amenable to treatment with autologous HSC. Allogeneic transplantation is basically always performed with curative intent.

Allogeneic Donor Selection

HLA matching has been the primary consideration when selecting an allogeneic donor [52]. The Class I molecules HLA-A, B, C and the Class II molecules HLA-DR ± DQ are considered in screening potential donors. In general, an HLA-matched sibling donor is considered the first choice. Because individuals inherit one set of HLA genes from each parent, any sibling has a 25% chance of being matched to the patient [53]. In addition, phenotypically matched related donors are identified for about 1% of patients, and somewhat less than 1% of patients will have an identical (syngeneic) twin donor [54]. Syngeneic donors, however, do not provide a GVT effect.

For patients lacking a matched sibling, an alternative donor must be identified. The typical second choice is an unrelated donor matched at high-resolution (DNA level) typing for at least 8 or 10 HLA antigens. Due to the efforts of organizations such as the Anthony Nolan Appeal in the UK, the National Marrow Donor Program in the United States, the DKMS in Germany, and other organizations worldwide, more than 25 million volunteer donors have been added to registries as potential HSCT donors [55]. Computer searches of those databases very quickly generate an idea as to the probability of finding an HLA-matched unrelated donor. However, the logistics of confirming eligibility and coordinating timing of an unrelated donor HSCT may take weeks to months, and this delay may be unacceptable for disease control in patients with aggressive malignancies [56]. The likelihood of finding an optimal donor differs between racial and ethnic groups, with the highest probability among whites of European descent, at 75%, and the lowest probability among blacks of South or Central American descent, at 16% [53, 57].

For patients lacking a matched unrelated donor, alternative donor options include mismatched unrelated donors, UCB, and HLA haploidentical donors. A rapidly increasing body of data supports the efficacy of UCB transplantation for patients lacking suitably matched related or unrelated donors. UCB units mismatched at one or two HLA loci and of sufficient size are available for almost all patients younger than 20 years of age and for more than 80% of patients 20 years of age or older, regardless of racial and ethnic background [57]. HLA-haploidentical donors—related donors who share only one haplotype (the maternal or paternal #6 chromosome) with the patient—are appealing, because nearly all patients will have a donor who is immediately available. HLA haploidentical donors are increasingly utilized as source of stem cells because of lower acquisition cost, availability regardless of race, fast procurement of stem cells, and the likely availability of the donors to collect additional cells if needed. Haploidentical transplantation outcomes have improved primarily because of the use of posttransplantation cyclophosphamide for GVHD prevention [58].

The Type of Hematopoietic Stem Cells

Once a donor is identified, a final consideration is whether to use HSC collected from bone marrow or peripheral blood; in some cases, cord blood might be the first choice.

Bone Marrow vs. Peripheral Blood

Bone marrow was the original source of HSC used for transplantation. Demonstration of the ability to mobilize HSC into the peripheral blood either with high-dose granulocyte colony-stimulating factor (G-CSF) or chemotherapy ± glucocorticoids made it possible to use peripheral blood stem cells (PBSC) for HSCT [59–61]. PBSC show a lower retention of rhodamine 123 (which is typical for noncycling cells), and may have a lower expression of CD38 as compared to bone marrow cells. Unless T cell depleted, PBSC contain significantly greater numbers of T cells than marrow.

PBSC sustain hematopoiesis as well as cells aspirated from the marrow. Many patients have now been followed for more than 15, 20, or even 25 years and show normal hematopoiesis [62, 63]. Given the ease of collecting PBSC, the proportion of PBSC transplants has increased significantly, and PBSC are currently used for basically all autologous transplants in patients with hematologic malignancies.

Numerous randomized trials have compared outcomes with bone marrow and PBSC. A recent phase III, multicenter, randomized trial of transplantation from unrelated donors showed no statistically significant difference in 2-year overall survival. The incidence of graft failure was significantly lower in the PBSC group, 3%, versus 9% in the bone marrow group ($p = 0.002$). The incidence of chronic GVHD at 2 years was significantly higher with PBSC, 53% versus 41% in the bone marrow group ($p = 0.01$). There were no significant between-group differences in the incidence of acute GVHD or relapse [64].

In patients prepared with RIC regimens, PBSC have been used almost exclusively as the source of HSC, in part because of the greater "engraftment potential" ascribed to those cells and in part related to the larger numbers of cells that are obtainable along with higher T-cell doses [65].

However, in patients undergoing HSCT for nonmalignant disorders, concern about the increased risk of GVHD associated with PBSC still favors the use of marrow [65]. Patients with nonmalignant diseases are unlikely to derive any gain from chronic GVHD.

Umbilical Cord Blood

Umbilical cord blood (UCB) has emerged as an important source of HSC. Since the first UCB transplant in 1988 using an HLA-identical sibling's UCB [66], there has been a steep increase in the use of UCB. The proliferative potential of UCB stem cells is greater than that of adult marrow or PBSC [67]. UCB contains proportionally higher numbers of progenitor cells than adult blood and possibly adult marrow [19, 68, 69]. Cord blood T cells are less immunocompetent than adult cells [66, 69]. Intracytoplasmatic signaling (following T-cell receptor engagement) in cord blood T cells differs from that in adult T cells [70]. Cord blood monocytes express lower levels of HLA-DR, CD86, and ICAM-1 than monocytes in adult blood, and produce lower levels of IL-10 and IFN-γ. IL-4 and IL-5 are basically absent [71]. These differences may contribute to the tolerability of greater HLA mismatching between donor and patient.

Additional advantages of cord blood include the ready availability (frozen off-the-shelf product), the absence of risk to the donor, and the minimal risk of transfer of viruses. Major challenges include the limited number of HSC and progenitors in a single cord unit, which can lead to delayed engraftment and graft failure—particularly in adults and large children—as well as possible delays in immune reconstitution. However, recent advances, including increased availability of units with a higher cellular content, the ability to use two units to increase cell dose, and various ex vivo expansion methods, have further increased the application potential of this cell source [72, 73].

Harvesting and Infusing HSC for Transplantation

Bone Marrow

Bone marrow is the primary organ of hematopoiesis in adults and the site from which stem cells for transplantation were traditionally harvested [74]. For that purpose, the donor (or patient) receives anesthesia, and under sterile conditions, multiple small volume aspirates (≈3 mL) of marrow are obtained from both posterior iliac crests [75]. Additional potential aspiration sites are the anterior iliac crests, the sternum, and, particularly in children, the tibia heads. Approximately 10–15 mL/kg donor weight is collected, usually yielding $1–4 \times 10^8$ cells/kg recipient weight (Table 55.3). The concentration of cells is generally higher in children than in adults and in first harvests than in subsequent aspirations if done within a short time interval after the first procedure [76–78]. The marrow is passed through filters, in closed systems, to break up cell aggregates and remove bone particles. If no ABO incompatibility exists and if the marrow is not to be subjected to any in vitro purging procedure or other manipulation, the resulting cell suspension is infused intravenously, generally via an indwelling intravenous catheter (e.g., Hickman line), and cells "home" through the peripheral blood circulation to the marrow cavity to reconstitute the hematopoietic system.

Table 55.3 Autologous and allogeneic peripheral blood stem cell (precursor) harvesting

	HSC source	
	Autologous	Allogeneic
Methods of mobilization	Chemotherapy, cytokines/growth factors (antibodies to adhesion molecules)	Cytokines/growth factors
Types of chemotherapy	Cyclophosphamide; etoposide; paclitaxel; epirubicin; ifosfamide; cisplatin; carboplatin (combinations)[a]	–
Cytokines/growth factors used	G-CSF (5–20 μg/kg/day)	G-CSF (2–20 μg/kg/day)
	GM-CSF (4–64 μg/kg; 125–250 μg/m²)	
	IL-3 (5–10 μg/kg)	
	TPO/MGDF (0.03–5 μg/kg)	
	SCF (10–25 μg/kg) (combinations)[b]	
Risk of tumor cell contamination	Yes (higher without chemotherapy)	No
Time course for harvest	Predictable for cytokines; variable for chemotherapy	Predictable

[a]Various schedules, generally disease-dependent, have been used; blood leukocyte and CD34+ counts are monitored for optimum timing of apheresis

[b]Various injection and infusion schedules are used. G-CSF, currently the most widely used regimen, is generally given once a day s.c. for 5 days with leukapheresis on day 5 (and day 6 if necessary) [79].

Abbreviations: *G-CSF* granulocyte colony-stimulating factor; *GM-CSF* granulocyte macrophage colony-stimulating factor; *HSC* hematopoietic stem cells; *SCF* stem cell factor; *TPO/MGDF* thrombopoietin/megakaryocyte growth and development factor

Peripheral Blood Stem Cells

At steady state, HSC circulate at very low concentrations in peripheral blood. Following cytotoxic therapy, however, the frequency of early hematopoietic precursors in the circulation increases dramatically, though transiently, during the recovery phase [59]. The same effect is achieved if cytotoxic agents such as cyclophosphamide, etoposide, or taxol are given for the very purpose of "mobilizing" cells from the marrow into the circulation (Table 55.3) [80]. The administration of recombinant hematopoietic growth factors, such as G-CSF, granulocyte-macrophage colony-stimulating factor (GM-CSF) or c-kit ligand alone or in combination, has similar effects (although presumably via different mechanisms [81]), and the timing of the maximum stem cell yield, around 4–6 days after initiation of treatment with growth factors, is more predictable than with chemotherapy [60, 61]. Furthermore, the absence of cytotoxicity and the associated side effects allows the application of this approach to healthy donors. The mechanism involved in dislodging cells from the marrow and forcing them into circulation is incompletely understood. HSC are normally anchored within the marrow environment, through specific receptor interactions with other cells and extracellular matrix. In agreement with that notion, plerixafor, an inhibitor of the stem cell homing receptor CXCR 4, has been shown to improve mobilization and do so within a shorter time interval [82].

PBSC (including cells at various stages of differentiation) are harvested from blood by leukapheresis. The best currently available marker of the cells required for a successful transplant (stem cells; long-term repopulating cells) is the surface glycoprotein, CD34. For autologous procedures the goal is to harvest approximately 3–5 × 10^6 $CD34^+$ cells/kg recipient weights [83]. Higher cell numbers are targeted if the cells are to be processed in vitro or if cells for a "back-up" infusion or second transplant are desired. The yield of $CD34^+$ cells per apheresis (usually 10–16 L over 2–4 h) for autologous transplants varies considerably, largely dependent on the intensity of prior therapy given to the patients. Lenalidomide-treated patients, for example, are difficult to mobilize, and a "lenalidomide-free" interval should be allowed before attempting cell collection.

The most reliable indicator of the likely success of apheresis is the concentration of $CD34^+$ cells in blood [84]. In many patients, one single harvest will suffice; in others, several sessions are required. However, after 2 (or 3) days of apheresis, generally the incremental cell yield of $CD34^+$ cells from additional aphereses is small. Large-volume apheresis may be useful in some patients. Usually autologous cells need to be cryopreserved until completion of the patient's conditioning regimen. Mobilization of PBSC by G-CSF in healthy allogeneic donors results in more consistent cell yields than in pretreated patients. By and large, 2–20 × 10^6 $CD34^+$ cells/kg are obtained with a single harvest [62, 63]. As the harvest can be timed such that it coincides with completion of the transplant conditioning regimen in the patient, allogeneic PBSC are usually transplanted fresh. Cryopreservation is possible, however, and has been used, for example, to accommodate donor preferences.

Cord Blood Cells

Cord blood is collected either by venipuncture in the third stage of labor while the placenta remains in utero or by gravity drainage of the ligated umbilical vein once the

placenta has been delivered [85]. Collected cord blood must be shipped to a bank for further processing and cryopreservation. Units are screened for size, and appropriate units are processed. A minimum size threshold for further processing is approximately 0.9×10^9 total nucleated cells. Processing strategies include directly freezing the unit in 10% DMSO vs. various approaches involving red blood cell and plasma depletion. Units may be manually depleted using hydroxy ethyl starch or PrepaCyte-CB or by automated systems including Sepax (Biosafe, Eysins, Switzerland) and AutoExpress (AXP; Thermogenesis). Though direct freezing results in the smallest loss of total nucleated cells, the large product volumes occupy significant storage space, can cause hemodynamic complications upon infusion in small patients, and result in potentially larger DMSO exposure of patients. RBC and plasma-depleted products reduce these concerns, but some cell loss is inevitable during processing [86].

The thaw and wash technique developed by Rubinstein et al. is most commonly used in preparation for infusion [87]. A thaw and dilute procedure may increase efficiency and potentially improve infused cell doses [88].

More than 600,000 UCB units have been stored for transplantation worldwide, and more than 30,000 UCB transplants have been performed. Cord blood is generally donated to a public cord blood bank, but private biobanks are expanding rapidly both in size (ViaCord, which in 2000 stored only a few thousand UCB units, held over 300,000 units in 2013) and in number. In 2011, approximately 1 million UCB units were stored in more than 130 private UCB banks worldwide [89]. Private biobanks are prohibited in some countries, such as Italy [90]. Given current indications for cord blood transplantation, uses of privately banked cord blood are extremely limited. Additionally, private banks are not currently regulated, and the quality of units collected is not guaranteed. Expert opinion does not currently encourage private cord blood banking [91].

Fetal Liver Cells

Fetal liver, if obtained at the optimum time of gestation, provides a rich source of HSC [92]. However, this stem cell source has become obsolete.

Clinical Data

Currently more than 40.000 transplants are carried out annually worldwide (57% autologous and 43% allogeneic). Transplant activity is increasing, more so in Eastern European countries than in the West, and there is a progressive increase in the use of HLA haploidentical family donors (by 25%), while the growth of unrelated donor HSCT has slowed, as has the use of CB. The main indications for HSCT are leukemias (33%; 96% allogeneic); lymphoid neoplasias (57%; 11% allogeneic); solid tumors (4%; 3% allogeneic); and nonmalignant disorders (6%; 88% allogeneic).

Autologous Transplantation

Malignancies

Multiple myeloma (MM) remains the leading indication for high-dose chemotherapy and autologous stem cell transplantation (ASCT) worldwide [93] and the International Guidelines recommend that ASCT be offered at some point during the treatment program for all medically fit patients [94, 95]. High-dose melphalan at 200 mg/m^2 is the standard conditioning for autologous transplantation (ASCT) with very low transplant-related mortality (TRM) [96, 97].

ASCT is also a standard treatment for patients with chemosensitive relapsed or refractory aggressive B-cell Non-Hodgkin Lymphoma, including diffuse large B-cell lymphoma (DLBCL), occurring de novo or via transformation of indolent disease. ASCT is also widely employed in patients with relapsed follicular lymphoma and forms part of primary therapy in those with mantle cell lymphoma [98]. Second-line salvage high-dose chemotherapy and ASCT are established therapy for refractory/relapsed Hodgkin lymphomas, leading to durable responses in approximately 50% of relapsed patients and a minority of refractory patients [99].

In acute leukemia, in contrast to MM or lymphomas, ASCT occupies more controversial place [100]. In fact, the optimal post-remission treatment for acute myeloid leukemia in first complete remission remains uncertain. Controlled prospective trials comparing consolidation chemotherapy with ASCT [101–103] and biological randomization to HLA-identical matched sibling donor myeloablative transplantation conducted in the mid-1990s favored the allogeneic bone marrow strategy because of a lower probability of relapse, despite the high incidence of treatment-related mortality. With improved conditioning regimens and the broad availability of allogeneic donors, ASCT does not play a significant role in the treatment of leukemia [104–107].

Autoimmune Disorders

While not part of the "Neoplastic Diseases," several autoimmune disorders, including multiple sclerosis, scleroderma, rheumatoid arthritis, and others have been treated successfully by HSCT, generally using autologous cells [108, 109].

CAR-T Cell Therapy

Strategies of ex vivo transfer a therapeutic gene (via a viral vector) into autologous HSC are now being integrated into

clinical trials. The ability to engineer T cells to recognize tumor cells through genetic modification with a synthetic chimeric antigen receptor (CARs) has ushered in a new era in cancer immunotherapy. The most advanced clinical applications are in targeting CD19 on B cell malignancies. This has become possible by the introduction of genes that encode high affinity tumor-targeting T cell receptors (TCRs) or synthetic chimeric CARs, respectively [110, 111].

The most impressive clinical results have been achieved in patients with refractory B cell malignancies including acute lymphoblastic leukemia, chronic lymphocytic leukemia, and lymphoma who have been induced into complete remissions after infusion of autologous T cells modified with a CD19-specific CAR [112–116]. The group from the Children's Hospital of Philadelphia and the University of Pennsylvania reported treatment of 53 children and young adults (≤24 years) with B-ALL who received $1.0–17.4 \times 10^6$ CAR-T cells/kg after lymphodepletion chemotherapy. Forty-five of 53 patients (85%) achieved marrow CR by flow cytometry at 1 month and 2 additional patients were in CR by 3 months without further therapy [116, 117]. They reported CD19 CAR-T cells effectively eliminated disease even in the CSF. Of the 45 patients in CR at 1 month, 20 subsequently relapsed, 13 with CD19-negative disease, and relapse-free survival was 44% at 12 months. However, CD19 CAR-T cell infusion may be associated with significant toxicities, including a cytokine release syndrome (CRS, with fever, hypotension, capillary leak, coagulopathy, and neurotoxicity). Seizures and stroke like phenomena have occurred. Interleukin-6 plays a major role in the development of toxicity, and the anti-IL-6 receptor (IL-6R) antibody, tocilizumab, and corticosteroids have been used therapeutically and pre-emptively. In most patients CRS and neurotoxicity are transient. The optimal approaches are still being determined.

While effective in many patients with B-ALL, NHL, and CLL, for unknown reasons other patients do not respond, even though they experience the same toxicities. Thus, major challenges remain.

Allogeneic Transplantation

Complications associated with allogeneic HSCT include graft failure, delayed engraftment, regimen-related toxicity, GVHD, infection, and relapse. Clinical data regarding each of these issues as well as general outcomes with respect to donor source are discussed later and summarized in Table 55.4.

Table 55.4 Complications of transplantation by transplant type

Donor source/ conditioning regimen	Graft failure	GVHD	Infection	Relapse
Autologous	[b]	N/A	[b]	[b]
Allogeneic	↑		↑	↓
MRD	[b]	[b]	[b]	[b]
MURD	[b]	↑	↑	↓
MMURD	↑	↑↑	↑↑	↓↓
Cord	↑↑	[b]	↑↑	? ↓↓↓[a]
Haploidentical	↑↑	↑	↑↑	↑
High-intensity	[b]	[b]	[b]	[b]
Reduced-intensity	↑	Similar (later onset)	? ↓	↑

[a]Double unit cord blood transplantation may be associated with particularly low relapse rates
[b]Reference value
GVHD graft-vs.-host disease, *N/A* not applicable, *MRD* matched related donor, *MURD/MMURD* matched/mismatched unrelated donor

Transplantations from HLA-Matched Related and Unrelated Donors

While HLA-matched sibling donors have inherited the same genes, HLA-typing at the *allele level* is required to determine true HLA identity in unrelated patient/donor pairs since the same serologically defined *antigen* can be shared by an entire family of alleles. In a large retrospective study, Lee et al. investigated the impact of HLA mismatching detected by low- or high-resolution DNA testing at HLA-A,-B,-C-DRB1 on outcome of unrelated allo-SCT. They concluded that 8/8 matching was the minimal level of matching required for transplant since matching for only 7/8 or 6/8 was associated with a 10%–15% decrease in 1-year survival with each incremental mismatch [118].

The CIBMTR retrospectively investigated the outcome of adult patients who received a myeloablative allo-SCT from either an 8/8 matched unrelated donor ($n = 941$) or an HLA-matched related donor ($n = 3158$) [119]. Unrelated transplant recipients showed a higher incidence of or treatment-related mortality (TRM) and relapse in cases of acute myeloid leukemia (AML), but not in those with acute lymphoblastic leukemia (ALL) or chronic myeloid leukemia (CML). In addition, leukemia-free survival (LFS) was lower for patients with AML who received 8/8 matched unrelated donor compared to the ones receiving grafts from related donors. This was not the case for patients with ALL or CML.

A prospective study from the French Society of Bone Marrow Transplantation and Cellular Therapy (SFGM-TC) investigated the outcome of 236 consecutive patients with standard-risk malignancy. The study included 55 recipients, less than 55 years of age with an unrelated donor matched for 10/10 alleles and 181 recipients with HLA-matched related donors.

Diagnoses included acute leukemia (*n* = 175), chronic myeloid leukemia (*n* = 43), and myelodysplastic syndrome (MDS; *n* = 18). All patients were required to receive the same myeloablative conditioning regimen and GVHD prophylaxis. The use of antithymocyte globulin (ATG) was not allowed. In multivariable analysis, overall survival and transplantation-related mortality were adversely influenced by recipient cytomegalovirus-positive serology, age of donor older than 37 years, and the occurrence of acute grade ≥ II GVHD. The effect of donor type was nonsignificant for OS, EFS, and relapse [120].

In patients with high-risk or advanced hematologic malignancies the Seattle team observed no significant differences between recipients of HLA-matched related and 10/10 matched unrelated donors, while in patients with intermediate risk disease TRM was higher and survival lower with transplants from 10/10 matched unrelated donors after myeloablative conditioning and PBSC transplantation [121]. If no 10/10 matched donor is available, transplant physicians have to select between mismatched donors. Petersdorf et al. reported an outcome analysis in 4796 unrelated donor transplants receiving myeloablative conditioning. Of those, 61% were 10/10 matched and 39% were mismatched for a single allele or antigen. After adjusting for disease stage, age, and ethnicity, the hazard of mortality conferred by a single HLA mismatch (any locus) was significantly higher than the hazard observed after matched unrelated transplants [122]. Fürst et al. reported on 2646 patients. All were high-resolution–typed for HLA-A,-B,-C,-DRB1, and -DQB1. The highest mortality was seen for HLA-A,-B, and -DRB1 mismatches. HLA-DQB1–antigen mismatched cases showed a nonsignificant trend toward higher mortality [123].

HLA Haploidentical Transplantation

Although high transplant-related mortality, mostly due to delayed immune reconstitution, has been a barrier to success in HLA haploidentical transplantation, outcomes have significantly improved with the advent of high-dose posttransplantation cyclophosphamide (PTCy) [124, 125]. PTCy has been now incorporated in several myeloablative and non-myeloablative conditioning regimens, including Flu with busulfan and thiotepa [126], Flu with melphalan and thiotepa or TBI [127], and Flu with ablative TBI doses [128]. Using these approaches relapse rates for patients with myeloid malignancies have varied from 20% to 40% at 1 year. In some studies, outcomes of haploidentical transplants performed with PTCy have been similar to those with HLA-matched transplants (related and unrelated) [129, 130]. These findings were recently confirmed in a large retrospective analysis of the Center for International Bone Marrow Transplant Research (CIBMTR). This study compared outcomes in patients with AML transplanted from an HLA haploidentical donor or an 8/8 HLA matched unrelated donor and showed comparable survival at 3 years for patients after myeloablative (41% vs. 42%, *P* = 0.87) or RIC/non-myeloablative conditioning (35% vs. 37%, *P* = 0.89) [131]. Haploidentical transplantation is an area of active investigation with novel approaches focused on better controlling alloreactivity, elimination of posttransplantation immunosuppression, prevention of disease relapse, and improvements in immunologic reconstitution.

Cord Blood Transplantation (CBT)

UCB transplantation has made HCT available to many more patients, and novel strategies such as ex vivo stem cell expansion have yielded encouraging results [72, 73]. Improved survival in adult CBT followed the observation that cell dose was critical for engraftment and survival, leading to studies on double CBT [132]. However, recent results have questioned the benefit of double as opposed to single CBT. In adults, a Eurocord retrospective analysis reported improved DFS for leukemia patients in CR1 receiving a double as opposed to single CBT but no advantage for patients in CR2 [133]. These data suggest that single CBTs may be appropriate for most children while the data for adults requires further investigation [134].

Investigators at the Fred Hutchinson Cancer Research Center and the University of Minnesota compared outcomes in 128 patients who received myeloablative conditioning and double CBT with the outcome of patients who received transplants from HLA matched related (*n* = 204), matched unrelated (*n* = 152), or mismatched unrelated donors (*n* = 52) [135]. Disease-free survival at 5 years was similar for the three groups, whereas the risk of relapse was lower in recipients of double CBT. Similar findings were observed in two subsequent smaller studies [136, 137].

Major obstacles to greater success of CBT include delays in engraftment and immune reconstitution that lead to increased mortality [138, 139]. Several methods to improve the speed of engraftment and decrease transplant related mortality are under investigation, including ex vivo expansion with cytokine cocktails, modification of homing, and the concurrent use of mesenchymal stem/stromal cells [72, 73, 140, 141].

Summary and Outlook

The field of HSCT has expanded exponentially in recent years. During the last decade, RIC transplant approaches have emerged as an effective strategy to treat older and more

infirm patients who could not previously benefit from HSCT. The use of "alternative" sources of HSC has facilitated clinical applications of this modality and has made it widely available. Recognition of then immunotherapeutic aspect of HSCT has led to exciting new developments such as the use of CAR T cells and other genetically modified donor cells.

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56 HLA Typing in Support of Hematopoietic Cell Transplantation from Unrelated Donors

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Introduction

Advances in the field of immunogenetics of the human leukocyte antigen (HLA) system have changed the landscape of unrelated hematopoietic cell transplantation (HCT). The advent of molecular tissue typing methods provided an unprecedented level of accuracy in the characterization of the class I and II genes and the much needed tools for querying the genetic diversity and implications of human genetic variation on clinical outcome after unrelated donor HCT. Clinical application of DNA-based typing methods for registry donors, together with the growth of international donor registries, has greatly increased the chances that any given patient may identify a suitable donor and benefit from a life-saving transplant.

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The HLA System

The HLA system was first described by Jean Dausset in 1952 after the discovery of agglutination reactions in recipients of blood products from multiparous female donors or blood donors who themselves were sensitized from previous blood transfusions [1]. HLA alloantigens were defined by serological methods in a complement-dependent microcytotoxicity assay using panels of alloantisera containing HLA antibodies that demonstrated the polymorphic nature of the HLA system [2–4]. Nomenclature was formalized starting in 1964 by the establishment of collaborative workshops [5]. The term "antigen" in HLA tissue typing describes epitopes that are recognized by antibodies, to be distinguished from epitopes that can be recognized by T cells. Transplantation responses involve both humoral (B cell) and cellular (T cell) immunity; although the link between HLA antigens and humoral responses is strong, the link between HLA alleles and cellular (T cell) responses is still an area of intense investigation, since not all allelic polymorphisms result in a T-cell response.

The HLA genes are located within a 4 Mb region at p21 on chromosome 6 within the major histocompatibility complex (MHC) and consists of three regions termed class I, class III, and class II. Genes within the class I and class II regions are structurally and functionally related; genes within the class III region, including tumor necrosis factor and heat shock protein, do not directly function as major transplantation genes but participate in the immune response through inflammatory reactions.

HLA Class I Genes

The class I HLA-A, B, and C genes lie within a 2 megabase region comprising the telomeric half of the MHC. Each class I gene is composed of 8 exons that encode specific domains of the class I molecule: exon 1, leader peptide; exon 2, α1 domain; exon 3, α2 domain; exon 4, α3 domain; exon 5, transmembrane

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domain; and exons 6, 7, and 8, cytoplasmic domain. The lengths of the exons are known to be the same for each serologically defined antigen. Polymorphism is most extensive in exons 2, 3, and 4, which form the basis for defining the HLA-A, HLA-B, and HLA-C alleles currently recognized [6]. Of the three extracellular domains of the class I molecule, the α1 and α2 domains fold to bind peptide in a groove between two α helices on a platform of a β-pleated sheet. The membrane-proximal α3 domain encodes the main binding site for CD8 and contact residues for β2 microglobulin [7, 8]. The extraordinary polymorphism of class I genes modulates the range of bound peptides and the T-cell repertoire expressed by CD8 T cells, the response to natural killer (NK) cells, and interactions of HLA-A, B, and C with peptide transporters (TAP) and chaperones [9–15]. The allele and locus-specific patterns of polymorphism of class I genes are thought to reflect distinct selective pressures exerted on the class I loci due to both specific antigenic challenge and the need for heterozygosity as a way of maximizing the range of antigenic responses.

HLA Class II Genes

Nine HLA genes reside in the class II region and share structural and functional similarity. The HLA-DR region encompasses five loci: HLA-DRA, DRB1, DRB3, DRB4, and DRB5. The HLA-DQ and DP regions encompass two loci each: DQA1, DQB1 and DPA1, DPB1, respectively. The class II molecule is a heterodimer of an α chain product of the A gene and a β chain product of the B gene. Within the HLA-DR gene, DRA and DRB1 products form the heterodimer that defines the HLA-DR1-DR18 serological phenotype. HLA-DRB3, DRB4, and DRB5 encode supratypic specificities that are expressed with DRB1 on only three haplotypes: DRB3 (DR3, DR11, DR12, DR13, DR14), DRB4 (DR4, DR7, DR9), and DRB5 (DR15, DR16). Haplotypes that are DR1, DR10, or DR8-positive contain only the DRA and the DRB1 genes. The DRA, DQA1, and DPA1 genes show limited diversity, whereas the DRB1, DQB1, and DPB1 genes are highly polymorphic [6].

HLA Typing Methodology

Definition of HLA class I and class II alleles has been made possible by the development of DNA-based typing methods over the last three decades. These techniques have had a significant impact on the ability to match potential stem cell donor and recipients with precision. The birth of the DNA typing era came in 1987 when the 10th International Histocompatibility Workshop demonstrated the utility of restriction fragment length polymorphism (RFLP) techniques to uncover polymorphism within serologically defined HLA-DR antigen families [16].

The advent of polymerase chain reaction (PCR) in the late 1980s revolutionized the HLA typing world, providing a new generation of powerful methods for allele typing [17, 18]. The major technological advances stemming from this prolific period of research include sequence-specific primers (SSP), sequence-specific oligonucleotide probes (SSOP), sequence-based typing (SBT), reference-strand conformational analysis (RSCA), oligonucleotide arrays, and, most recently, next-generation sequencing (NGS).

Sequence-Specific Primers

Complete sequence homology of the template DNA with the PCR primers allows maximal efficiency and specificity of the PCR amplification reaction; mismatching of the DNA template and primer for one or more nucleotide bases causes inefficient amplification. SSP methodology utilizes panels of PCR primers that are descriptive of known HLA polymorphisms [19–25]. Panels of primers can be informative for DNA substitutions that distinguish families of HLA antigens ("group specific") or that are unique for specific alleles ("allele specific"). After the test DNA is amplified using the entire panel of PCR primer pairs, the product(s) is electrophoresed in a gel. The presence of an amplified product is indicative that the test DNA encoded the same sequence as the PCR primer (Fig. 56.1). The HLA genotype is determined by evaluating the pattern of positive and negative amplifications to the entire primer panel. The SSP method is a cost-effective technique whose main advantage is simplicity and rapidity for typing at the equivalent level of the HLA antigen family ("low resolution" or "intermediate resolution" level typing). Definition of the allele ("high resolution") generally necessitates large panels of primer pairs; hence, SSP methods for high-resolution typing are more labor intensive and less cost effective than the probe-based technologies described in the next sections.

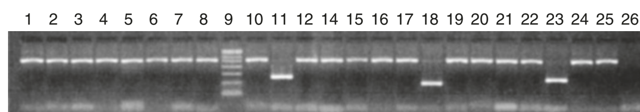

Fig. 56.1 Sequence-specific primer (SSP) technique demonstrating positive PCR amplication for HLA-C*07 in lane 11 and for HLA-C*12 in lanes 18 and 23. Lanes containing a single PCR-amplified band are controls; molecular weight marker was loaded in lane 9. Methodology was previously described by Bunce and Welsh [21]

Sequence-Specific Oligonucleotide Probes

Sequence-specific oligonucleotide probe (SSOP) methods were among the earliest tools developed for typing polymorphic positions of HLA alleles [26–30]. The first protocols were designed using radiolabeled probes and hybridization of each probe to the target DNA. Several generations of SSOP methods were subsequently developed that incorporated strips of probes and nonradioactive labels. The newest platforms use bead technology and chemoluminescence for detection of hybridization (http://www.onelambda.com). The basic principle of SSOP relies on the design of oligonucleotide probes to specific regions within the polymorphic exons of class I genes (exons 2, 3, and 4) and of class II genes (DRB, DQA1, DQB1, DPA1, DPB1). After the test DNA is amplified by PCR, the amplified product is allowed to hybridize to the SSOPs. Only those probes having complementary sequence with the test DNA will hybridize; SSOP that are mismatched will fail to hybridize. The presence of hybridized SSOP to DNA is signaled by the label (Fig. 56.2). The HLA sequence of the template DNA is deduced by the pattern of positive and negative hybridization. Panels of SSOP can be descriptive of every nucleotide position of the exon; alternatively, SSOP panels may be informative for only certain positions that are known to be polymorphic. The use of the latter, while informative for known alleles, would not detect novel alleles encoding substitutions outside of the probed regions. SSOP techniques are well suited for high-resolution typing and are especially efficient for large-scale analysis.

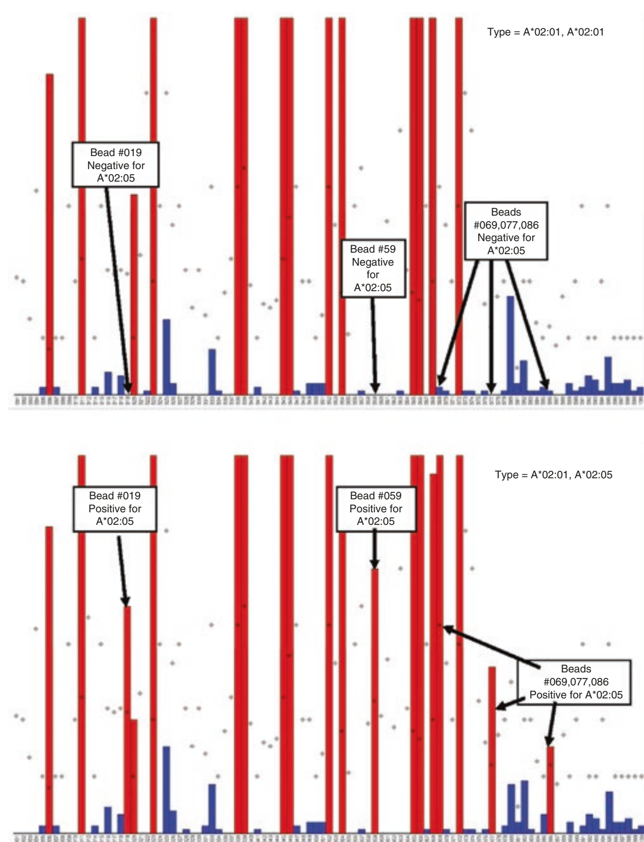

Fig. 56.2 Representative typing using the Luminex® bead-based technology (http://www.onelambda.com). *Top panel* shows the reactivity pattern of HLA-A*02 beads for a homozygous A*02:01, 02:01 sample. Each *bar* represents the reaction of a bead to the template DNA, with the *blue bars* indicating no reactivity (A*02:05) and the *red bars* indicating positive reactivity (A*02:01). *Bottom panel* shows the results for an A*02:01, 02:05 heterozygous sample (Samples courtesy of Dr. Shalini Pereira)

Array Technology

Oligonucleotide array technology has revolutionized the study of molecular biology. Its applications have been far-reaching including large-scale gene discovery, gene expression monitoring, detection of mutations and polymorphisms, and mapping. The HLA system represents an ideal application of this particular technology because of the highly polymorphic nature of the class I and II genes [31]. Array methods allow parallel analysis of simultaneous HLA gene polymorphisms. It is a flexible method in which the addition of new probes descriptive of novel substitutions may be easily configured onto the array. The use of fluorescent labels allows ease in the application of this method to a high-throughput clinical laboratory. Finally, the results of the hybridization can be quantified. An approach for sequence analysis of HLA-B has been recently described [31]. This system utilizes a panel of oligonucleotide probes designed for all known polymorphisms in exons 2 and 3 of the gene. The presynthesized probes are arrayed onto glass slides. Following PCR amplification of HLA-B using fluorescent-labeled primers, the PCR product is allowed to hybridize with the array; stringent conditions are required to discriminate perfectly matched duplexes and detect a single nucleotide difference between the template DNA and the probe (Fig. 56.3). The array is scanned using an automated fluorescent scanner, and the HLA-B sequence is determined by quantitative analysis of the hybridization pattern. Oligonucleotide array technology has its greatest potential in the scalability and reduced cost for assaying large numbers of samples.

Sequence-Based Typing

Sequence-based typing (SBT) methods provide information on the nucleotide sequence of the template DNA and permit detection of single base differences to enable discrimination of unique HLA alleles. Its power lies in it being a highly robust, accurate, and definitive technique for uncovering novel variation [32–36]. Many approaches for SBT have

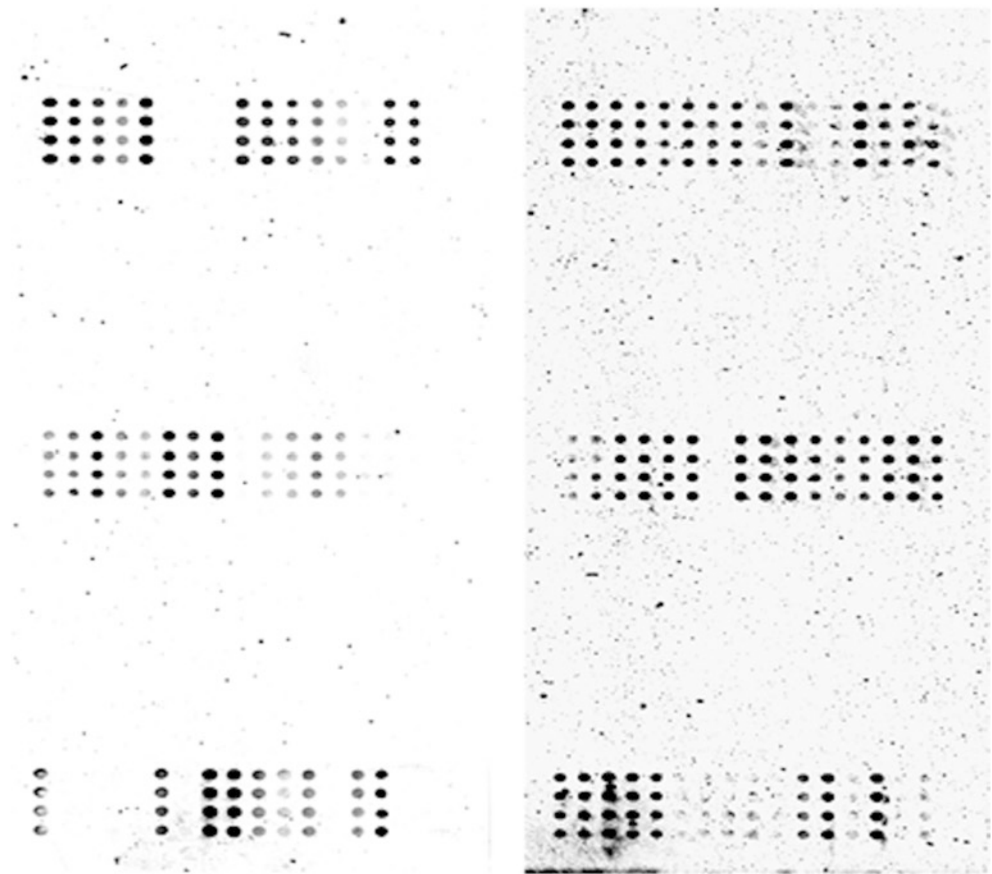

Fig. 56.3 Oligonucleotide array for HLA-B. Actual hybridization of array using probes for HLA-B, reviewed in Guo et al. [31]

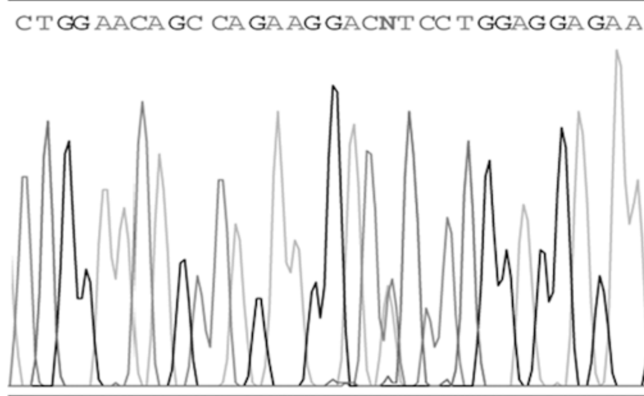

Fig. 56.4 Sequence-based typing (SBT) method demonstrating chromatogram for HLA-DPB1*03:01, 04:01-positive sample using methodology published by Rozemuller et al. [34] and Verslius et al. [36]. Chromatogram demonstrates incorporation at "N" of both C and A at position 182 of exon 2 corresponding to DPB1*03:01 and DPB1*04:01, respectively

been developed including direct sequencing from PCR-amplified genomic DNA or from cDNA. Among the most widely used methods is automated fluorescent sequencing. In this method, after the HLA allele(s) is PCR amplified, cycle sequencing is performed using fluorescent-labeled primers. Alternatively, sequencing can use fluorescent-labeled dideoxynucleotides. The sequenced templates are electrophoresed in a polyacrylamide gel in an automated sequencer. The fluorescent signals emitted from either the labeled primers or the labeled dideoxynucleotides are detected by a laser within the sequencing apparatus and interpreted by software (Fig. 56.4). Automated allele identification using software simplifies and automates the data analysis.

The physical linkage of markers on the same chromosome, also known as phase, is an essential feature of unambiguous assignment of alleles, particularly when an individual expresses two different HLA alleles ("heterozygous") [37, 38]. Using HLA class I proteins as an example, HLA-A, B, and C are defined by sequence polymorphisms in exons 2, 3, and 4, which span 1.7 kilobases inclusive of introns. When both alleles are co-amplified together, the inability to phase across multiple exons and introns will generate a mixture of sequences that could be represented by more than 1 combination of alleles. For traditional Sanger sequencing-based methods, the phase of polymorphisms can be achieved by designing amplified templates that overlap at each end so that the full-length phased sequence can be assembled.

New methods surmount not only the need for long template reads that can be sequenced, but also the phase across extremely long distances. Known as "next-generation sequencing" or NGS, these automated sequencing platforms permit phasing of complex sequence polymorphisms across

long stretches of HLA genes. The ability to assay multiple regions of genes at the same time, and in many samples simultaneously ("sequencing in parallel") offers an entirely new level of DNA sequence ascertainment. Because of its ability to define phased sequences, NGS is also known as "clonal sequencing."

One of the earliest NGS platforms was "454" sequencing by Roche, in which clonal amplification is combined with on-phase sequencing [39]. This technology used long-range PCR amplification of the entire class I gene, and fragmented the template for sequencing, followed by assembly of the sequences for definitive allele assignments. The use of beads to capture single-stranded DNA molecules was a revolutionary strategy that offered a powerful approach for phasing complex variation in HLA genes. After the advent of the prototype 454 sequencing method, several other platforms have been developed. For example, the "MiSeq" protocol by Illumina is based on dye terminator chemistry and uses primers that are immobilized on a chip [40]. Ion Torrent is bead-based and uses hydrogen emission for detection. The PacBio "SMRT" (single molecule real-time sequencing) system uses clonal sequencing of single molecules and can sequence up to 10–15 kilobases of template [41]. Another variation of SBT is represented by whole exome sequencing where the entire genome or exome sequence spanning 4–5 million basepairs and multiple HLA genes is targeted [42]. Current protocols may provide typing for HLA-A, C, B, DRB1, DRB3, DRB4, DRB5, DQA1, DQB1, DPA1, and DPB1 genes in a single sequencing run. A clear advantage of NGS methods over traditional sequencing is that millions of single DNA molecules are sequenced in parallel at a significantly lower cost than conventional sequencing, and as such, these methods are highly amendable for typing large numbers of registry donors at recruitment [43]. Regardless of the specific sequencing platform, throughput and phasing capabilities, NGS strategies all require informatics to facilitate data analysis (please refer to the section below entitled "Information Technology in Support of Unrelated Donor Identification").

Imputation of HLA Genotype with Single Nucleotide Polymorphisms (SNPs)

The imputation of HLA genotypes from SNPs has been an invaluable tool for ascertaining the most probable HLA tissue type in samples that have been characterized with dense SNP arrays for the HLA region [44]. Imputation is the inference of HLA types from SNP genotype data. The SNPs used for imputation do not typically reside within the HLA genes themselves. Rather, the SNPs are located inter-genically. Imputation relies on positive linkage disequilibrium (LD) between SNPs and the nucleotide substitutions in exons that define the HLA type. HLA imputation is most commonly used for SNP genotyped research samples that have never been HLA typed. Recent studies have also shown that imputed KIR alleles correlate with sequenced KIR alleles [45].

HLA Nomenclature

The origins of HLA nomenclature date back to the third workshop when the designation "HLA" was introduced: *H* referring to the Hu1 system described by Dausset, and *LA*, the designation given by Payne and Bodmer for the same antigen system, discovered independently in the two laboratories [1, 46]. "HLA" emerged as an acronym for "human leukocyte antigen." The naming of new HLA alleles is the responsibility of the World Health Organization Nomenclature Committee for Factors of the HLA System (http://www.ebi.ac.uk/imgt/hla/). With the universal application of molecular-based tissue typing methods, novel HLA sequences are being discovered at a rapid rate. Beginning on April 1, 2010, a new nomenclature was adopted to permit unlimited numbers of new alleles to be named. Following the term HLA, the locus is specified as -A, -B, -C, -DRB1, -DQB1, etc. An asterisk (*) signifies that the name is derived from DNA. Following the asterisk, the serologic equivalent of the antigen, the allele number, silent substitutions, and differences outside of coding regions are given in order, each delimited by a colon (:). The name is completed by information as to the level of expression, if known. For example, the name HLA-A*24:02:01:02L refers to the A24 serologic antigen equivalent (24), the second allele of the A24 family (02), the first silent substitution (01), intron polymorphism (02), and low-expression variant (L). As of June, 2016, over 3399 HLA-A, 4242 HLA-B, 2950 HLA-C, 1883 DRB1, 911 DQB1, and 644 DPB1 alleles are officially recognized (www.imgt/hla).

Information Technology in Support of Unrelated Donor Identification

With the growth of unrelated stem cell donor registries worldwide, and the increasing complexity of the HLA system, there has been a worldwide effort to develop the necessary information technology to aid clinicians and patients identify the most suitable donor for transplantation and support the coordination of the procurement of donor stem cells between registries and transplant centers [47]. These efforts represent a multidisciplinary approach by experts in the fields of HLA immunogenetics, bioinformatics, programming, clinical transplantation, and registry operations. The availability of powerful NGS typing strategies now in place in many clinical HLA laboratories has in particular demanded a new level of data management and tools for the exchange of HLA information within the clinical community. MIRIN is a recently organized consortium intended to facilitate downstream analysis and data management of HLA data. Included among the research and

development activities are the development of a checklist that enables the content of NGS genotyping results to be evaluated and compared across genotyping platforms. The ability to reanalyze historic HLA typing data in the context of sequencing data generated by NGS methods with modern nomenclature is a major goal of the consortium. This goal has immediate utility in bridging legacy HLA typing with primary sequenced data generated by NGS methods [48].

Tissue Typing in Support of Unrelated Donor HCT

The Early Years

Experience with the use of volunteer unrelated donor HCT began in the early 1970s. The preliminary results demonstrated the high-risk nature of these transplants, especially with respect to graft failure and GVHD [49–59]. In addition to the medical needs and complications faced by recipients of unrelated donor transplants, the practical issues of identifying suitable donors dominated the early experience, and lengthy, expensive donor searches limited its application more broadly.

Major progress was made in the 1980s and 1990s in allogeneic transplantation with respect to improved supportive care of transplant patients, particularly the development of effective antiviral agents to prevent and treat cytomegalovirus (CMV) infection and the development of immunosuppressive regimens for the prophylaxis of GvHD [60–62].

Recognition of the importance of HLA compatibility between the donor and recipient in mediating host-versus-graft and graft-versus-host reactions, together with the growth of unrelated donor registries worldwide, contributed to successful use of unrelated volunteer donors for HCT [63–68]. The first volunteer donor registry was organized in London at the Anthony Nolan Research Centre, clearly demonstrating public interest in volunteer donation as well as the financial feasibility of performing registry HLA typing [69]. In the United States, the National Marrow Donor Program (NMDP) was established in 1986 [70] and was followed soon thereafter by Bone Marrow Donors Worldwide [71]. The network of international donor registries is coordinated by the World Marrow Donor Association (WMDA) [72] and includes donor registries and 350 donor centers representing 52 countries. This collaborative effort represents over 28 million volunteer donors (www.worldmarrow.org).

The DNA Era

The advent of DNA-based typing methods has provided powerful tools for understanding the biological relevance of allele disparity in unrelated donor HCT. The elucidation that a single HLA antigen can be encoded by many unique alleles has had enormous clinical implications for the typing and selection of appropriate donors for transplantation. It is clear that DNA-based methods, when applied to the analysis of serologically matched patients and donors, can uncover allelic disparity [73–79] and are also more reliable than alloantisera [28, 29, 80]. Increased sensitivity and specificity of DNA-based methods compared to serologic reagents were validated in a quality control study by the National Marrow Donor Program (NMDP) [29]. In a single center analysis of 300 CML unrelated donor transplants, sequence-based typing (SBT) methods for HLA-A, B, and C genes and SSOP methods for HLA-DRB1 and DQB1 were used to define alleles; 47% of these pairs were matched for all five genes, and 53% were mismatched for at least one class I or class II allele [81]. A large retrospective analysis by the NMDP evaluated the rate of undetected HLA disparity in 1259 patients who had received HCT from unrelated donors identified by the NMDP [75]. This study utilized SSOP and SBT methods to analyze DRB, DQA1, DPA1, and DPB1. Only 9.4% of the 1259 pairs were found to be matched at all loci tested. Noteworthy were the findings that 79.4% were matched for DRB1, but only 13.2% for DPB1. Sixty-six percent of the study population encoded mismatches for two or more class II alleles. Worldwide experience has confirmed the power of DNA methods to uncover donor–recipient allele disparity among putative well-matched pairs. DNA-based methods should therefore be used as the gold standard for identifying HLA class I and class II alleles in support of donor selection for unrelated donor HCT. Continued refinements in methodology including the use of filter paper spots as a robust source of high-quality DNA permitted the application of large-scale HLA typing for registry typing [82].

Today, the integration of NGS technologies for donor registry typing has provided laboratories with an unprecedented level of throughput as well as a vast new body of sequence data for both coding and noncoding regions of HLA genes [43]. As described below in the section entitled “HLA Expression,” the clinical significance of noncoding regions has only just begun to be elucidated. The emergence of technologies that readily capture the complete sequence of HLA genes provides investigators with the much needed tools for investigating the functional impact of noncoding regions.

Clinical Impact of Donor HLA Incompatibility

The probability of identifying an HLA-compatible donor depends on many factors including the patient’s ethnicity and the size and composition of donor registries [83]. When only mismatched donors are available, clinicians can be better equipped to avoid donors whose HLA mismatch may

increase risks to the patient. Today, the tools available to clinicians and search specialists are based on the level of resolution of the HLA mismatch (allele or antigen), the presence of mismatching for specific amino acid residues, and the level of expression of the patient's and the donor's mismatched HLA allotypes.

The Vector of Incompatibility

The early haploidentical related HCT experience served as an important platform for understanding the role of HLA in unrelated HCT and provided the clinical and immunogenetic basis for the definition of the vector or direction of HLA mismatches [63]. Nonshared HLA antigens of the donor could provoke host-versus-graft (also known as the "HVG" or "rejection vector") allorecognition leading to significantly increased risk of graft failure [84]; furthermore, graft failure was compounded by the presence of a positive pretransplant cross-match [63]. HLA mismatching with graft-versus-host allorecognition (also known as the "GVH vector") was also shown to influence risk of clinically severe acute GVHD after haploidentical donor HCT [85–87].

The original observations in the early haploidentical transplant experience have subsequently been validated in several large independent studies. In a recent analysis by the Center for International Blood and Marrow Transplant Research (CIBMTR), patients transplanted from unrelated donors with a single HLA-A, B, C, or DRB1 mismatch in the HVG vector had a lower risk of GVHD than patients receiving transplants from donors with a single HLA disparity in the GVH vector [88]. These results confirm that HVG vector mismatches are well-tolerated with respect to GVHD, and that the direction of incompatibility has biological consequences.

The Effect of the Number and Nature of HLA Mismatches

The early studies directed at understanding donor HLA mismatching analyzed patient–donor pairs who were variably typed at HLA-A, C, B, DR, and/or DQ, using a combination of serological and DNA-based typing methods [89–94]. Hence, interpretation of the data requires knowledge of the limitation of the typing technologies that were available at the time of each study. The first locus for which outcomes data were available was HLA-DRB1 followed soon thereafter by HLA-DPB1, and later HLA-DQB1. Information on the importance of class I was feasible once SSP, SSOP, and sequencing methods were designed and optimized; tools for HLA-A and B preceded those for HLA-C [76, 79, 81, 93, 95–100]. Despite these technological limitations, the early literature clearly established the concept that (1) DNA-based methods could detect allele mismatches among serologically matched transplant pairs, (2) allelic differences were functional, and (3) increasing numbers of allele mismatches correlated with increased risks.

The availability of DNA methods for all six classical HLA loci, together with an expanded unrelated donor clinical transplant experience, led to the current recommendations for donor typing and matching for HLA-A, C, B, DRB1, DQB1 alleles, the "10/10" matched donor [73, 76, 96, 99–106]. Today, transplantation from a 10/10 allele-matched donor yields outcomes comparable to HLA identical sibling transplantation for certain patient groups [107].

Most recently, the CIBMTR has shown the deleterious effect of additive donor–recipient disparities at the supratypic class II loci termed HLA-DRB3, DRB4, and DRB5 [108]. HLA-DRB1 is in strong positive LD with each of these supratypic genes when DRB3/4/5 are present according to the DRB1 haplotype as described in the preceding section entitled "HLA Class II Genes." When a patient and donor are mismatched at HLA-DRB1, there is a high likelihood that the pair is also mismatched at DRB3/4/5. In the retrospective CIBMTR analysis [108], as the number of mismatches at DRB1/DRB3/DRB4/DRB5 increased, the risk of mortality increased. From a donor selection standpoint, when an HLA-DRB1-mismatched donor is considered, typing of DRB3/DRB4/DRB5 (where relevant) is indicated to define risks to the patient.

Recently, the Japan Marrow Donor Program (JMDP) evaluated the incidence of acute GVHD according to the number of mismatches at HLA-A, B, C, DRB1, DQB1, DPB1 as defined by high-resolution molecular methods [109]. Higher risks of acute GVHD and overall mortality were observed as the number of donor–recipient mismatches increased from 1 to 5, compared to no HLA mismatching. Interestingly, not all HLA mismatches conferred the same risks. A single HLA-DRB1 or HLA-DQB1 mismatch was not associated with higher risks of GVHD or mortality, but when patients and donors were mismatched at both HLA-DRB1 and DQB1, risks increased significantly. These data suggest that HLA-DRB1 and DQB1 could be defined as a "super-gene" complex. When the study population was reanalyzed according to DRB1/DQB1 mismatches, the total number of HLA-A, B, C, DPB1, and DR/DQ mismatches was associated with increased risks of grades III-IV acute GVHD and overall mortality.

The tally effect of multi-locus HLA mismatches has recently been evaluated by the CIBMTR [110]. In a series of over 10,000 unrelated donor transplants, increasing numbers of HLA mismatches correlated with worse outcome. Non-HLA factors were also identified as risk factors. Survival was better for patients with younger age donors (aged 18–32 years). For every 10-year increment in donor age, the

hazard of mortality increased by 5.5%. Sex, parity, and CMV serostatus were not associated with survival. The JMDP and CIBMTR studies support the selection of a young donor with the fewest HLA mismatches when a fully compatible donor is not available.

The sixth HLA locus, HLA-DPB1, also functions as a classical transplantation gene, because donor HLA-DPB1 mismatching is associated with higher risks of graft failure and GVHD [96, 99, 105, 111–117] and a lower risk of disease recurrence after HCT, suggesting a potential beneficial effect of graft-versus-leukemia ("GVL") activity [111, 113, 117, 118]. Although the clinical importance of donor matching for HLA-DP was shown after the effects of other classical class I and II genes were already integrated into routine clinical HLA testing of unrelated donors, new information on the importance of this locus has enabled further refinements in donor selection to be made, as described in more detail in the sections below entitled "Donor Mismatching for Class II Residues" and "HLA Expression."

Donor Mismatching: Alleles Versus Antigens

The molecular typing methods described earlier in this chapter provide the means to detect single nucleotide differences between the patient and donor. Differences detectable only by molecular tools discriminate between two unique alleles ("allele" mismatches). When differences between the patient and donor HLA molecules can be detected by alloantisera (i.e., serological methods), the mismatches are referred to as "antigen" mismatches, as they define epitopes of the expressed HLA molecule that can be recognized by the humoral immune system.

Are allele and antigen mismatches functionally different? Historical studies suggested that antigen mismatches are more immunogenic than allele mismatches [73] particularly for HLA-C [98]. These data suggested that when HLA-matched donors are not available, prioritization of allele mismatched over antigen-mismatched donors may decrease the risk of posttransplant complications. Whether these concepts could be applied equally to each HLA gene has been an important area of investigation, not only for donor selection but also for understanding the characteristics of HLA molecules that are immunogenic. In an early study by the CIBMTR, antigen and allele mismatches conferred similarly high risks with one important exception, HLA-C [103]. When compared to transplants from HLA-A, B, C, DRB1 allele-matched donors ("8/8"), HLA-C allele mismatches ("7/8") were not associated with statistically significantly higher mortality, TRM, or acute GVHD; however, transplantation from donors mismatched for HLA-C antigens conferred increased risk for mortality and acute GVHD. These data suggested that selection of an HLA-C allele-mismatched donor should be prioritized over an HLA-C antigen-mismatched donor, when HLA-matched donors or single DQB1-mismatched donors are not available.

New information has become available on the most common HLA-C mismatch observed among unrelated donor–recipient pairs, the HLA-C*03:03/03:04 combination [119]. This particular mismatch does not elicit in vitro cytotoxic T cell responses. Patients mismatched for the C*03:03/03:04 allele combination had similar outcomes as HLA 8/8-matched recipients [119]. A possible basis for the lower risks associated with this particular mismatch compared to other HLA-C mismatches, has recently been elucidated and is described below in the section entitled "HLA Expression."

The immunogenicity of HLA class I mismatches may depend on the locus that is mismatched. An analysis by the NMDP [120] found no correlation of the specific combinations of HLA-A allele mismatches with engraftment, acute or chronic GVHD, relapse, TRM, or survival. It was estimated that over 11,000 donor–recipient pairs would be required to fully address whether specific HLA-A allele mismatch combinations are more or less deleterious.

Most of the information regarding the clinical significance of donor HLA mismatching and outcomes has come from marrow grafts. In 2008, over 19,481 adult stem cell donations were provided to patients worldwide, and of these, 7260 were peripheral blood stem cell (PBSC) products [72]. The increasing use of mobilized peripheral blood stem cell (PBSC) products for transplantation motivated a new analysis of these data by the CIBMTR [121, 122]. An increased step-up in risks associated with allele and antigen mismatching was observed compared to the historical bone marrow transplant experience. Furthermore, only HLA-C antigen mismatches were significantly associated with mortality [122]. This study suggests that the effect of donor HLA may depend on the manipulation of the stem cell product. With the vast majority of patients now receiving peripheral blood stem cell grafts [123], more information is needed to clarify the difference of locus-specific effects after PBSC compared to bone marrow transplantation.

HLA-Mismatched Transplantation Today: Reduced-Intensity Conditioning

Although the concepts of HLA matching in unrelated donor transplantation grew out of the clinical experience with traditional ablative conditioning regimens, data also support the importance of donor–recipient HLA mismatching with reduced intensity conditioning regimens. In a study of patients receiving reduced-intensity conditioning for unrelated donor transplants, patients with an HLA 8/8-matched donor had improved 1 and 3 year survival rates compared to HLA 7/8-matched transplants [124]. Transplantation from a

7/8-matched donor was associated with higher risks of grades II-IV acute GVHD and transplant-related mortality, and lower probabilities of disease-free survival and overall survival. No differences in risks of chronic GVHD or relapse were appreciated. These data are consistent with those from experience with ablative conditioning regimens and suggest that additive effects of HLA mismatching should be avoided where possible.

Donor Mismatching for Key Residues of Class I Molecules

Molecular HLA typing methods can discriminate single nucleotide substitutions that permit unique HLA alleles to be identified and distinguished from closely related allele sequences. These DNA sequences in turn represent amino acid sequences of the mature HLA protein. Most substitutions in HLA molecules are located within the peptide binding groove and/or at positions involved in contact with the T-cell receptor. In unrelated donor HCT, the earliest report of HLA mismatching and outcomes demonstrated that even a single amino acid difference between the recipient and donor HLA-B genotype could increase risk of graft failure [125]. This initial observation of class I disparity and graft failure was later borne out by a single center study that demonstrated the role of HLA-A, C, and B allele-level mismatches on graft failure and suggested that the location of putative high-risk amino acid residues might define patients at highest risk for graft failure [98].

The importance of mismatching at specific residues for different amino acids has been explored in different models. The cytotoxic T lymphocyte precursor (CTLp) assay has been employed to test the hypothesis that alloreactivity arises from donor recognition of recipient sequence polymorphisms encoded in the α1 and α2 domains of class I molecules [126]. Lower CTLp frequencies are associated with a lower probability of GVHD [127]. CTLp reactivity is a measure of the number of mismatches encoded within the α helix and β-pleated sheet of single class I locus mismatches [128]. The presence of 5α/5β mismatches in addition to a negative CTLp was a strong predictor of favorable outcome compared to any other mismatch, suggesting that both the number of mismatched epitopes and the ability of these epitopes to elicit a CTLp provide additional information than either variable alone in assessing whether a given HLA class I mismatch may be permissible.

Comparative sequence analysis has provided novel information on putative residues important in GVH allorecognition. The earliest study to correlate transplant outcome with mismatching for specific class I residues identified mismatching at positions 114 and 116 in HLA-B-mismatched transplants to be predictive of severe GVHD and high TRM [129]. The observations from this Italian analysis were extended in a large-scale study by the Japan Marrow Donor Program (JMDP) [130]. A total of four HLA-A, one HLA-B, and seven HLA-C mismatch combinations (along with two HLA-DR/DQ haplotypes and two HLA-DP nonpermissive mismatch combinations) were significantly associated with transplant outcomes. Risks increased with increasing numbers of nonpermissive mismatches. Donor–recipient mismatching at seven specific positions correlated most strongly with GVHD risk: Tyr9–Phe9 of HLA-A and Tyr9–Ser9, Asn77–Ser77, Lys80–Asn80, Tyr99–Phe99, Leu116–Ser116, and Arg156–Leu156 of HLA-C. These early data suggested that HLA-A and HLA-C locus mismatches that do not involve the high-risk amino acid disparities may be permissible.

A role for donor mismatching for residues 116 and 99 of HLA-C, and residue 9 of HLA-B has recently been validated by the CIBMTR [131]. Donor mismatching for amino acids at residue 99 of HLA-C was associated with higher risk of transplant-related mortality, and mismatching at residue 116 was associated with a significantly higher risk of acute GVHD compared to matching at these residues. Donor mismatching at residue 9 of HLA-B was associated with higher risk of chronic GVHD compared to matching at residue 9. These recent data confirm the importance of high-resolution typing and matching at key residues that influence the repertoire of peptide antigens that bind in the groove between the alpha helices of class I molecules, thereby influencing outcomes after HCT.

Donor Mismatching for Class II Residues

HLA-DP has served as a model locus for understanding the importance of donor matching for class II residues. Site-specific mutagenesis has been used to elucidate HLA-DP motifs that define alloreactive T-cell epitopes (TCEs) [112, 116]. Presence of the 8LFQG11 motif in the HLA-DP β chain was found to correlate most strongly with T-cell responses. As many as 75% of donor–recipient pairs are not mismatched for this TCE disparity in the host-versus-graft direction, and use of such donors would be predicted to decrease the risk of graft failure, especially in the setting of HCT for thalassemia [116].

The concept of permissible HLA-DP epitopes has been explored recently by the International Histocompatibility Working Group (IHWG) in HCT [117]. This study tested the hypothesis that (mis)matching for immunogenic TCEs encoded by particular HLA-DPB1 alleles may be relevant to outcome after unrelated HCT. In this model, mismatching in both the graft-versus-host (GVH) and host-versus-graft (HVG) vectors was analyzed. HVG mismatching may include the potential for the mismatched donor antigen presenting

cells to cross-present allogens of the patient to donor T cells, thereby increasing the risk of GVHD. Mismatches associated with higher risks are called “nonpermissive,” to be distinguished from mismatches associated with low cytotoxicity (“permissive mismatches”). In a population of 5838 HLA-A, C, B, DRB1, DQB1 matched transplants, 4490 were DPB1 mismatched and 1348 were DPB1 matched (“12/12”). The HLA-DP-mismatched pairs were defined as TCE-matched or TCE-mismatched. Compared to the DP-mismatched TCE-matched group, the DP-mismatched TCE-mismatched group had higher risks of grades III–IV acute GVHD, TRM, and mortality, but lower relapse (see section below entitled “Beneficial Consequences of HLA Mismatching”). The 12/12 transplants had lower TRM and severe acute GVHD, but higher relapse. The IIHWG observations were confirmed in an independent cohort of patients by the CIBMTR [132]. These results suggest that mismatches involving DPB1 alleles carrying the same TCE might be well tolerated, whereas mismatches involving a TCE disparity confer risk. A tool to score patient and donor DPB1 alleles according to TCE permissive and nonpermissive mismatches can be found at www.imgt.hla.

Beneficial Consequences of HLA Mismatching

Early clinical experience described an overall lower risk of posttransplant disease recurrence among patients who had experienced clinical acute GVHD [133]. Many studies have subsequently confirmed a relationship between GVHD and a reduced risk of relapse, a phenomenon known as the graft-versus-leukemia (GVL) effect. Historical data from the JMDP identified four HLA-C and six HLA-DPB1 allele mismatch combinations associated with lower risk of relapse, eight of which were allele combinations that differed from those previously identified for GVHD [111].

More recent studies have identified strong GVL effects associated with HLA-DPB1 mismatching [117, 134]. In the setting of T-cell depleted transplantation, concurrent mismatching for HLA-DP with other HLA loci was associated with better outcomes in patients with more advanced disease [134]. A new JMDP analysis of HLA-A, B, C, DRB1, DQB1, and DPB1 mismatched transplants [109] uncovered only HLA-C and HLA-DPB1 mismatches to be associated with a lower risk of relapse. Taken together, these observations suggest that patients with HLA-DP-mismatched donors might derive some benefit from GVL effects of the mismatch. Elucidation of the underlying mechanisms responsible for GVL involving T cell or natural killer cell-mediated effects remain an important area for research.

HLA Expression

Noncoding sequences can affect expression levels, and the information gleaned from regulatory regions of HLA genes may be leveraged to define permissive HLA mismatches. Outside of transplantation, HLA-C and HLA-DP expression each play an important role in the control of infection, HIV-AIDs for HLA-C and hepatitis B for HLA-DP [135, 136]. In addition, the level of HLA-DP expression correlates with the risk of Crohn’s disease [135], suggesting a potential role for HLA expression in autoimmunity. The theory behind HLA expression in disease relates to the presentation of foreign antigens, which may be more robust when larger numbers HLA molecules are expressed at the cell surface, resulting in improved immune surveillance. By the same token, high HLA expression may provide the target needed to elicit a strong autoimmune attack on host tissues leading to autoimmunity.

The recent observation made by the CIBMTR for cumulative effects of HLA-DRB1/DRB3/DRB5 mismatching described above may implicate a tally effect of several low-expression gene products on clinical outcome [108]. The level of expression of HLA-DRB3, DRB4, DRB5, DQB1 genes is generally lower than that of HLA-A, B, C and DRB1. Among HLA 8/8-matched transplants, a donor–recipient mismatch involving a single low-expression allele was not associated with higher risks compared to matching, but the presence of 3 or more low-expression mismatches at HLA-DRB1/3/4/5/DQ in addition to a mismatch at HLA-A, B, C or DRB1, was associated with higher overall mortality and transplant-related mortality.

The clinical significance of HLA-C and HLA-DP expression in outcome after unrelated donor hematopoietic cell transplantation has recently been investigated. Using the median fluorescent intensity value for each HLA-C allotype, each HLA-C mismatch in a population of HLA 9/10-matched donor–recipient pairs with a single HLA-C mismatch was used to measure the hazards of mortality and odds of GVHD [137]. As the level of expression of the patient’s mismatched HLA-C allotype increased, the risks of acute GVHD and non-relapse mortality also increased. HLA-C*03 and C*07 molecules have the lowest levels of expression, and C*01 and C*14 the highest levels of all HLA-C gene products [135]. Risks depended not only on the level of HLA-C expression, but also on residue 116 and KIR ligand epitope mismatching. Patients with low-expression HLA-C*03 and 07 mismatches that were also residue 116 and KIR ligand-mismatched, had similar clinical outcome as HLA-C-matched transplants. However, patients with high-expression HLA-C*01 or 14 mismatches that were also residue 116 and KIR ligand-mismatched had higher risks. These data strongly support a role for the level of HLA-C expression in immunogenicity related to graft-versus-host alloresponses.

In contrast to the allotype-specific expression patterns exhibited by HLA-C molecules, low or high level expression of HLA-DP gene products appears to be regulated by a polymorphism within the noncoding region, the 3′ untranslated region (UTR), of HLA-DPB1 [136, 138]. A recent retrospective analysis of HLA 10/10-matched patient–donor pairs found higher GVHD rates when the patient's mismatched DPB1 allele was a high-expression allotype, compared to patients with low-expression HLA-DPB1 mismatches [138]. These observations are consistent with the hypothesis that highly expressed mismatched patient HLA-DP allotypes may be potent targets for donor-anti-host recognition. The data furthermore illustrate the clinical importance of understanding diversity of regulatory regions of HLA genes, as a means to facilitate the selection of mismatched unrelated donors.

HLA Allele-Specific Risks in Transplantation

A rich history of clinical research in disease associations has elucidated a major role for HLA alleles in the susceptibility to a wide range of diseases [139]. In transplantation, the vast majority of information on HLA is founded on a mismatch model where the patient and donor encode different HLA alleles; however, recent information suggests that risks may also arise from the presence of specific alleles carried by either the patient or the donor. In an analysis of T-replete unrelated donor bone marrow transplants facilitated by the JMDP, patients and/or donors who possessed HLA-B*51:01 had significantly higher posttransplant risks than patients and donors who were B*51:01-negative [140]. Possession of certain HLA-C alleles also influenced the risk of GVHD and mortality in the mismatch model. Compared to HLA-C-matched patients, HLA-C-mismatched patients with an HLA-C*14:02 allele had the highest risk of severe acute GVHD transplant-related mortality of all patients in the series. These results suggest that feature(s) specific to the HLA-B*51:01 and HLA-C*14:02 allotypes may be involved in transplant-associated risks, including those that arise from donor recognition of these molecules by recipient cells.

The Role of the Noninherited Maternal Antigen

In Transplant Outcome

Noninherited maternal antigens (NIMAs) are those antigens of the nonshared haplotype among haploidentical siblings. For example, for an HLA-A1, A2-positive patient, an HLA-A1, A3-positive donor (mismatch is between recipient A2 and donor A3), and an HLA-A2, A3-positive mother of the donor, the noninherited maternal A2 antigen is matched to the patient's A2 ("NIMA matched"). The impact of NIMAs on the donor's immune system (and hence, the recipient) may be significant. The tolerizing effect of NIMAs has been observed in haploidentical related donor HCT under a variety of transplant regimens [141–143]. These studies have demonstrated that transplantation from mother to child is associated with low risk of both acute and chronic GVHD and mortality, indicating that there is donor-specific suppression of T-cell responses against NIMAs. In contrast, transplantation from father to child is associated with higher risk of GVHD, indicative of an immunizing effect of paternal antigens.

Proof of principle has recently been established with the demonstration of NIMA-associated protective effects in cord blood transplantation [144]. This retrospective study included 1121 patients who had received a single unit cord blood transplant for whom HLA typing of the recipient, the unit, and the mother of the baby was available. HLA typing at three loci—HLA-A, B, and DRB1—was evaluated. For each transplant pair, the number of mismatches in the patient that were identical to the noninherited maternal haplotype of the cord blood unit was scored. Of the 1121 total pairs, 1059 pairs were mismatched for one or two antigens at HLA-A and/or B and/or DRB1. Of these mismatches, the mismatched antigen(s) were shared between the transplant recipient and the mother of the cord blood unit in 79 (7%). Of these, 25 patients had one mismatch and 54 had two mismatches. Outcome after transplantation for patients whose mismatched antigen was shared with the NIMA of mother of the cord blood unit ("NIMA match") was compared to that for patients who did not share the NIMA of the mother of the cord blood unit ("NIMA mismatch"). Lower transplant-related mortality (TRM) was observed with NIMA-matched transplant compared to those who shared no NIMAs.

These data suggest that, in utero, the cord blood is exposed to the NIMAs, and this two-way trafficking of cells or soluble HLA antigens promotes tolerance of noninherited maternal histocompatibility antigens by the immune system of the fetus. When the transplant is performed with cord blood stem cells that have been tolerized to the NIMA, which happens to be the same antigen in the transplant recipient that is mismatched, there is less graft-versus-host allorecognition, leading to lower GVHD, lower TRM, and improved survival. These data have potential implications to the selection of potential cord blood units. If maternal HLA typing were available for cord blood units, further consideration to select the unit whose NIMA is the same as the HLA mismatch between the recipient and the cord blood unit might be envisioned. More data will be needed to ascertain the size of available cord blood registries that will optimally support the integration of NIMA information into cord blood selection. The relative risks associated with transplantation using a unit

with fewer HLA mismatches but without NIMA matching, compared to a lesser matched unit with NIMA matching, remain research questions of great interest.

Typing for the Human Platelet Alloantigen System

The technical developments made for typing the human platelet alloantigens (HPA) and glycoproteins parallel those that have been achieved for the HLA gene complex. Serological methods dominated the early history of platelet typing, with the first alloantigen, Zw[a], having been discovered using agglutination methods [145]. Between the 1950s and the 1980s, serologically based methods were refined and immunochemical techniques were introduced [146–151]. Representative techniques that have been widely used in the discovery of new HPA and in clinical testing include the platelet suspension immunofluorescence test (PSIFT) [151], the mixed passive hemagglutination test (MPHA) [150], the modified antigen-capture enzyme-linked assay (MACE) [147], and the monoclonal antibody-specific immobilization of platelet antigens (MAIPA) [148, 152].

The modern era of HPA typing has witnessed the development of PCR-based DNA typing methods that share similarity with those described previously for the HLA class I and II genes. The earliest application of molecular technology to HPA typing came in 1989 with the use of RFLP analysis for HPA-1 [153]. RFLP techniques were subsequently applied to investigate the polymorphism of HPA-2, 3, 4, and 5 [145, 154]. The development of SSP approaches [155, 156] and SSOP methods [157–159] was instrumental in uncovering the allelic diversity of the HPA system.

Large-scale HPA typing is currently feasible with high-throughput tools: oligonucleotide ligation assay (OLA), preferential homoduplex formation assay (PHFA), allele-specific PCR, melting curve analysis, and 5′-nuclease assays [160–164]. One of the most technically facile approaches for rapid cost-effective HPA genotyping is based on the TaqMan® oligonucleotide hybridization method relying on the 5′-endonuclease activity of *Taq* polymerase to cleave a dye fluorophore from the oligonucleotide probe. The genotypes are defined by the clustering of fluorescence for the different dye fluorophores. This simple read-out enables the user to rapidly identify samples that have homozygous or heterozygous genotypes.

The most challenging clinical scenario remains transfusion support of alloimmunized platelet-refractory patients. Approaches to facilitate the selection of HLA-matched platelet products are available, in addition to screening for anti-HLA antibodies [165]. Current and future research are addressing the benefits of HLA-matched or cross-matched compatible platelet components, and the utility of class I residue matching.

Conclusions

The success of unrelated donor HCT as a curative modality for hematologic malignancies is due in part to a better understanding of the immunogenetics of the HLA system and to the development and application of robust DNA typing technologies for donor matching. The extensive diversity in both the coding and noncoding regions of HLA genes has important biological implications in unrelated donor HCT. Whereas complete genotypic identity between a donor and recipient is associated with optimal transplant outcome, at least with respect to engraftment and risk of GVHD, it is clear that not every disparity is functionally relevant. Future clinical research will be required to define the acceptable limits of HLA disparity and to identify mismatches that are well tolerated so that life-saving transplants can be made available to patients lacking matched unrelated donors.

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57 Special Care of Blood and Marrow Hematopoietic Cell Transplant Recipient

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Introduction

Hematopoietic cell transplantation (HCT) has evolved into one of the most effective therapeutic modalities for the treatment of neoplastic, immunologic, and genetic disorders in pediatric and adult populations [1]. Careful preclinical scientific studies in the late 1940s and 1950s set the stage for the triumph of clinical HCT [2–4]. In the late 1960s and early 1970s, E. Donnall Thomas and his colleagues at the Fred Hutchinson Cancer Research Center refined clinical allogeneic bone marrow transplantation techniques sufficiently that cures could be obtained in far-advanced leukemia and aplastic anemia subjects [5]. By the 1980s, autologous bone marrow transplantation was demonstrated to provide long-term survival in acute myeloid leukemia and relapsed adult lymphoma subjects [6, 7]. These successful experiences helped move HCT to use earlier in the disease course, including patients in first complete remission but thought to be at high risk for subsequent relapse, and led to exploration in a variety of disorders.

HCT is a life-saving art and more than one million subjects have undergone the procedure [8]. It is estimated that more than 65,000 persons per year undergo HCT, with a 3% growth rate per year. Due to a greater understanding of human histocompatibility, advances in transfusion medicine, improved antibiotics, more effective anticancer drugs, and the development and implementation of recombinant hematopoietic growth factors, overall- and disease-free survival rates dramatically have improved. Alternative sources of hematopoietic grafts have been expanded and include, in addition to the use of autologous grafts, HLA-mismatched related and unrelated, HLA matched unrelated adult volunteers, HLA haploidentical and umbilical cord blood (UCB) cells. The use of UCB as a graft source is unique for its low number of hematopoietic progenitor cell content due to small volume yet highly proliferative and novel immunologic properties. UCB is an important graft source, especially in pediatric diseases [9–11]. Culture-expanded bone marrow stromal cells, termed multipotent mesenchymal stromal cells (MSCs), are being utilized in conjunction with hematopoietic cells to enhance engraftment, reduce the incidence and severity of graft-versus-host disease (GvHD), and facilitate organ repair [12–15].

The potential for serious morbidity and mortality of the myeloablative preparative regimen may preclude a number of patients from undergoing allogeneic transplant. These patients, however, could benefit from a HCT procedure and now may benefit from a “nonmyeloablative” or “reduced-intensity conditioning” regimen (Table 57.1). These approaches depend more upon cellular therapy via the allogeneic effect to eliminate neoplastic cells rather than the potential cytotoxic effect of chemoradiation on neoplastic cells. The phenomenon of donor cells eradicating tumor often is referred to as the graft-*versus*-cancer effect and appears to be driven by donor alloreactive T-cells [16–19]. Certain hematologic maliganancies are more vulnerable to this graft-versus-cancer effect than others [19]; the explanation for such variability is not clearly understood. Older patients and those with significant comorbid illnesses now are potential candidates for HCT whereas in the past these subjects would not be considered for conventional myeloablative HCT procedures due to the unacceptably high treatment-related mortality (TRM) [18]. The pioneering work of E. Donnall Thomas and his colleagues ultimately was recognized with the 1990 awarding of the Nobel Prize in Medicine/Physiology to Professor Thomas. Today, bone marrow transplantation has continued to undergo significant technological and clinical transformation. HCT, a term which encompasses autologous or allogeneic transplantation of hematopoietic progenitor cells from bone marrow, blood,

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Table 57.1 Definition of conditioning regimens for hematopoietic cell transplantation according to the intensity of chemotherapy

Intensity of conditioning regimen	Definition	Mechanism of effect
Myeloablative conditioning (MAC)	Regimen destroys the hematopoietic cells in recipient bone marrow with resulting long-lasting pancytopenia, usually irreversible. Fatal in most instances unless hematopoiesis is restored by infusion of HPCs	Potent cytotoxic agents and the allogeneic ("graft-versus-tumor") effect combine to eliminate malignancy and facilitate donor cell engraftment
Reduced-intensity conditioning (RIC)	Regimen intermediate in intensity between MAC and NMA. Results in pancytopenia that may be prolonged and requires HPC rescue	Modest intensity cytotoxic agents and the allogeneic ("graft-versus-tumor") effect combine to eliminate malignancy and facilitate donor cell engraftment
Nonmyeloablative (NMA)	Regimen results in minimal cytopenias but significant lymphopenia and does not require HPC rescue	Limited effect of cytotoxic agents; relies predominately on the allogeneic ("graft-versus-tumor") effect to eliminate malignancy and facilitate donor cell engraftment

or UCB, is an effective modality for the treatment of variety of neoplastic and nonneoplastic conditions. Presently, essentially all patients now have the possibility of undergoing either an autologous HCT or an allogeneic HCT, the latter using a related, histocompatible or partially mismatched donor; matched unrelated adult donor; an umbilical cord blood graft; or a HLA-haploidentical donor graft. Further, regimen intensity can be adjusted per patient tolerance, e.g., myeloablative conditioning; reduced-intensity conditioning; and nonmyeloablative conditioning. The age of recipients steadily has increased as well. As a result of continued improvements and sophistication of supportive care, an increasing number of transplants also are being undertaken in the ambulatory, rather than the inpatient, setting. In the US, the number of allogeneic transplants recently surpassed 8,000 per year, with a cumulative total of 320,000 transplants [20] (Fig. 57.1). Currently, more than 30,000 transplant recipients are surviving beyond 5 years. Should transplant survival rates remain unchanged, by 2030 there may be over half a million long-term survivors in the US, and up to one million worldwide [20, 21] (Fig. 57.2). The use of this modality, however, requires extremely sophisticated medical, financial, and psychological supportive care for a successful outcome [22, 23]. This review describes the medical aspects of HCT recipients in a detailed fashion.

Recipient Suitability

The effectiveness of myeloablative therapy and HCT must be tempered by the potential for significant morbidity and mortality [24]. The decision to proceed with HCT must take into consideration disease- (type and state of disease) and patient- (comorbid conditions) related factors since these variables influence all aspects (pre-, peri-, and post-) of transplant [25–31] (Table 57.2). Armand and Coworkers [25] developed the Disease Risk Index (DRI) after analyzing the outcomes of 13,131 patients reported to the Center for International Blood and Marrow Transplant Research (CIBMTR) who underwent HCT between 2008 and 2010. The DRI stratified patients into four risk groups (Table 57.2) with 2-year overall survival (OS) ranging from 64 to 24% and is the strongest prognostic factor, regardless of age, conditioning regimen intensity, graft source, or donor type. With

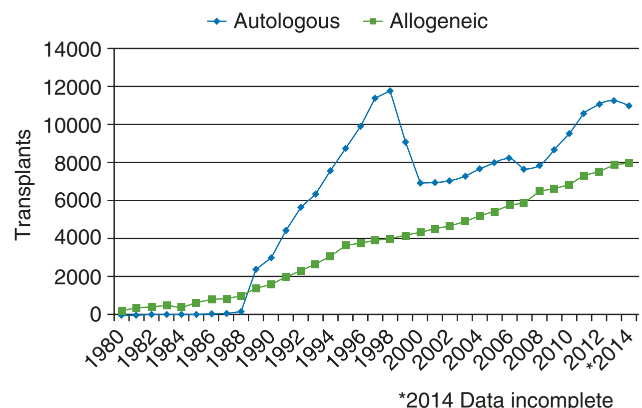

Fig. 57.1 Estimated annual numbers of transplant recipients in the U.S. were compiled according to the number first transplants registered with CIBMTR [20]

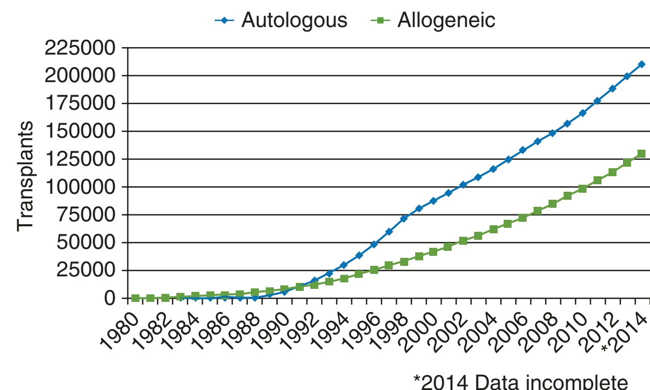

Fig. 57.2 Cumulative number of transplant recipients in the U.S. reached 340,000 in 2014 (CIBMTR data) [20]

Table 57.2 Disease risk index for allogeneic hematopoietic cell transplantation [25]

Disease risk group	2-year overall survival (%)	Disease risk category
Low	**66**	• Chronic lymphocytic leukemia or small lymphocytic lymphoma in complete or partial remission
		• Chronic myeloid leukemia in chronic phase
		• Acute myeloid leukemia[a] with favorable risk cytogenetics in complete remission
		• Hodgkin lymphoma in complete remission
		• Mantle cell lymphoma in complete remission
		• Clinically indolent B-cell non-Hodgkin lymphoma in complete or partial remission
Intermediate	**51**	• Chronic lymphocytic leukemia or small lymphocytic lymphoma in stable condition or progressive disease
		• Chronic myeloid leukemia in accelerated phase
		• Acute myeloid leukemia[a] with intermediate risk cytogenetics in complete remission
		• Acute lymphoblastic leukemia in first complete remission
		• Low-risk myelodysplastic syndrome[b] (<5% blasts and intermediate cytogenetics) in any disease stage
		• Low-risk myelodysplastic syndrome[b] (<5% blasts and adverse cytogenetics) in early disease stage
		• High-risk myelodysplastic syndrome[b] (≥5% blasts and intermediate cytogenetics) in early disease stage disease
		• Mantle cell lymphoma in partial remission
		• Clinically aggressive B-cell non-Hodgkin lymphoma in complete or partial remission
		• T-cell non-Hodgkin lymphoma in complete or partial remission
		• Multiple myeloma in complete or partial remission
High	**33**	• Acute myeloid leukemia[a] with favorable risk cytogenetics AND induction failure or active relapse
		• Acute myeloid leukemia[a] with intermediate risk cytogenetics AND induction failure or active relapse
		• Acute myeloid leukemia[a] with adverse risk cytogenetics AND complete remission
		• Acute lymphocytic leukemia in second or third complete remission
		• Low-risk myelodysplastic syndrome[b] (<5% blasts and adverse cytogenetics) with induction failure or active relapse
		• High-risk myelodysplastic syndrome[b] (≥5% blasts and intermediate cytogenetics) with induction failure or active relapse
		• High-risk myelodysplastic syndrome[b] (≥5% blasts and adverse cytogenetics) at any stage
		• Mantle cell lymphoma with induction failure or active relapse
		• Burkitt lymphoma in complete remission
		• T-cell non-Hodgkin lymphoma with induction failure or active relapse
		• Multiple myeloma- relapsed or refractory
Very-high	**23**	• Chronic myeloid leukemia in blast crisis
		• Acute lymphocytic leukemia with induction failure or active relapse
		• Aggressive B cell non-Hodgkin lymphoma with induction failure or active relapse
		• Burkitt lymphoma with induction failure or active relapse or partial response to therapy
		• Acute myeloid leukemia[a] with adverse cytogenetics AND induction failure or active relapse
Examples of risk assessment score systems for allogeneic hematopoietic cell transplantation		
	Attributes and caveats	**Score assessment**
EBMT [26] **(2009)**	Mainly for chronic myeloid leukemia patients.	Overall survival and treatment related mortality calculated based on assessment of 5 factors;
	Limited utility in current era	1. Donor type
		2. Stage of chronic myeloid leukemia
		3. Age of the patient
		4. Donor/recipient gender matching
		5. Time to transplant from diagnosis

(continued)

Table 57.2 (continued)

PAM [27] **(2006)**	Only useful for predicting the risk for death within the first 2 years after hematopoietic cell transplantation	The risk for death within 2 years for patients with PAM scores in the highest category was significantly higher than for those with scores in the lowest category
		Eight pretransplantation clinical variables:
		1. Patient age
		2. Donor type
		3. Disease risk
		4. Conditioning regimen
		5. FEV1
		6. Carbon monoxide diffusion capacity (DLCO)
		7. Serum creatinine concentration
		8. Serum alanine aminotransferase (ALT) concentration
HCT-CI [28] **(2005)**	Assessment of patients who have a variety of hematologic neoplasms	HCT-CI score weighs 17 different categories of organ dysfunction to calculate a score ranging from 0 to 29 for overall survival and nonrelapse mortality assessment
	Prospectively validated	Online web-based calculator available at www.hctci.org
	Age added to the index [32]	
	Most widely used	

[a]Acute myeloid leukemia t(8;21), inv(16), and t(15;17) were classified as favorable cytogenetics, complex karyotype ($>$4 cytogenetic abnormalities) as adverse cytogenetics, and all others as intermediate
[b]myelodysplastic syndromes, abnormal chromosome 7 and complex karyotype (≥4 abnormalities) were classified as adverse cytogenetics, and all others as intermediate.
Modified from Ref [25]
EBMT European Group for Blood and Marrow Transplantation, *PAM* Pretransplantation Assessment of Mortality, *HCT-CI* hematopoietic cell transplantation-specific comorbidity index

regard to patient-related factors and transplant outcomes, a number of composite scoring systems are available that may assist estimating mortality risk in patients considering HCT [26–31]. Of these, the three most commonly used assessments are the European Group for Blood and Marrow Transplantation (EBMT) risk assessment score; the Pretransplant Assessment of Mortality (PAM) score; and the Hematopoietic Cell Transplantation-specific Comorbidity Index (HCT-CI) (Table 57.2) [26–28, 32]. Advances in supportive care have not only improved short-term outcomes but have also resulted in many more long-term survivors and the need for continuing follow-up on this groups to prevent or intervene early for problems that may develop [31]. Additionally, the expanded use of reduced-intensity conditioning (RIC) regimens has allowed more patients formerly excluded from this procedure due to organ dysfunction to undergo successful allogeneic HCT. Defining the ideal conditioning regimen for more vulnerable patients, especially those who are considered "elderly" or with compromised organ function, remains a challenge. Recently, Sorror and coworkers proposed the integration of age into the comorbidities index for clinical decision-making and comparative effectiveness in the setting of allogeneic HCT [32]. Data from 3033 consecutive recipients of HLA-matched grafts from five institutions contributed to this analysis. The patients whose composite comorbidity-age index were low (scores 0 or 1–2) had comparable mortality risk regardless of conditioning regimen used; these subjects should be offered higher-intensity regimens. On the contrary, patients with higher scores (≥ 3) had statistically significant higher mortality risk and are more suitable for lower-intensity regimens. Although many investigators have examined the role of pretransplant evaluation in predicting organ-specific, treatment-related morbidity, few have focused on identification of pretransplant factors associated with mortality in older adults undergoing RIC-based HCT. McClune and colleagues [33] published a retrospective analysis of over 1000 patients age 40 years or older who underwent RIC allogeneic HCT. They examined the influence of age-group on several outcome measures including marrow recovery, acute and chronic GvHD and nonrelapse survival. Age had no significant impact on any outcomes using RIC regimens.

Optimal Donor for Allogeneic Hematopoietic Cell Transplant

Prospective allogeneic HCT candidates undergo histocompatible testing to identify HLA suitable hematopoietic cell donor [34, 35]. The HLA donor searches begin with family members; if a full HLA-match sibling is not identified, then the process is extended. Options include searches with

unrelated registries such as the National Marrow Donor Program for an HLA-match unrelated donor (MUD); or, institutional preference, for HLA-partially matched donors or HLA- haploidentical mismatched donors; or UCB grafts. Optimal donor characteristics include HLA identity at the A, B, C, and DRB1 loci, same gender for donor and recipient, younger donor age, same ABO red blood cell (RBC) type, and CMV concordance. Rarely are all such characteristics met. Priority is given to HLA- matching over other parameters. Often a family member donor is not identified as fully HLA- match and alternative types of grafts are sought. Allogeneic HCT performed using unrelated donors are associated with higher rates of graft failure, increased incidence and severity of GvHD, and increased susceptibility to infection when compared to HLA- matched sibling-donor transplants [36]. The development and application of DNA-based typing techniques for class I and II molecules for identifying histocompatibility between donor and recipient at the A, B, C, DR, and DQB1 loci (10/10 preferred allele match in unrelated donor setting) is a substantial improvement over serologic methods [34, 35, 37–40]. These sophisticated techniques have enabled selection of a more congruent graft resulting in fewer cases of graft rejection and has lowered the incidence and severity of GvHD. Inherited minor histocompatibility antigen mismatches, deriving from polymorphic peptides encoded elsewhere than chromosome 6 in the genome, are key molecules in the development of graft-versus-tumor and GvHD effect, especially in the HLA-matched transplant setting [41–43].

In addition, greater emphasis during donor selection now is being placed on specific NK cell alloreactivity [44] and DP locus status for improvement in transplant outcomes [45–51]. Recent data suggest that an HLA-DPB1 mismatch is associated with increased risk of graft failure and GvHD. The observation that the risks of GvHD may be offset by a beneficial effect of graft-versus-leukemia (GvL), leading to lower disease recurrence, provides a potential therapeutic avenue for patients with high-risk malignancies. Attempts to refine graft selection within the realm of 7 out of 8 HLA-match unrelated donor transplant setting has brought forth few intriguing concepts [52–56]. First, the transplant outcomes were not adversely affected if mismatch antigen (7 out of 8 HLA matched setting) was in the host-versus graft-direction (unidirectional HvG vector, i.e., the host immune system identifies the donor allele and mounts a reaction) [52, 53]. Second, adverse transplant-related outcomes were seen if ≥3 mismatches are present within DRB 3/4/5, DP, and DQ in GvH direction [54]. Third, the mismatch combination HLA-C*03:03–03:04 is shown to be clinically well tolerated due to lower risk associations with GvHD and mortality compared to other HLA-C mismatches; this effect was not accompanied by increased hazard of relapse [55]. Finally, mismatches involving high expression HLA-C alleles were shown to be associated with significantly higher risks of severe acute GvHD but not of relapse compared to low expression HLA-C mismatches [56]. These observations suggest that HLA-C*03:03-03:04 and low-expression HLA-C mismatches might allow for at least partial separation between GvL and GvHD. Few similar concepts appear to apply to HLA-DPB1 mismatches [49]. The process of locating a HLA-matched unrelated donor and securing a graft, however, may take up to many months, on average about 3 months. Depending on the ethnicity of the potential recipient, a HLA matched donor may not be located. A recent study from the NMDP showed that the likelihood of finding an optimally matched (8/8 match at HLA-A, -B, -C and -DRB1) donor ranged from 75% for patients of European descent down to 16% for Blacks of South or Central American descent [57]. Alternative donor sources also include HLA-haploidentical family members and UCB units (discussed later in text) providing a donor for almost 100% of the patients in need of allogeneic transplant [58]. The former are a ready source of hematopoietic cells, while the latter can be procured via the National Marrow Donor Program or via UCB banks such as the New York Blood Center. The incidence and severity rates of GvHD are lower with UCB units than with other hematopoietic cell sources, in part, due to the low numbers and immaturity of lymphocytes contained in the product [59, 60]. Specifically, recipients of UCB obtained from HLA-identical siblings had a lower risk of acute and chronic GvHD when compared to patients given bone marrow grafts obtained from HLA-identical siblings [61]. Other data demonstrate a lower risk of GvHD in recipients of unrelated donor UCB versus HLA-matched unrelated bone marrow [62]. On the other hand, UCB transplants, especially in adults, are hampered by a smaller number of cells in the graft (per recipient weight), longer duration of severe neutropenia, a higher graft failure rate, and the potential for increased treatment-related morbidity and mortality [60, 63]. In children, nonrelapse mortality is lower in allele level matched single UCB vs. mismatched units at 1, 2, 3, 4, or 5 alleles considering HLA-A, -B, -C, and -DRB1 loci and the myeloablative conditioning setting [64]. Transplantation of more than one unit and the recently developed expansion of UCB cells ex vivo may ameliorate these limitations [65]. Further, use of double UCB grafts may be associated with a lower malignancy relapse rate due to an enhanced GvL effect [66, 67]. The use of UCB grafts is discussed in greater detail elsewhere in this text.

Graft Source: Peripheral Blood versus Marrow

First utilized in the late 1980s, blood rather than marrow has become the preferred source of support for autograft and allograft patients alike. Many trials [68–87] have demonstrated that the use of mobilized blood grafts is associated

with faster time to return of neutrophil and platelet counts, fewer infections, fewer transfusions, shorter hospital stays, reduced antibiotic use, and often lower costs than its bone marrow counterpart. Also, the rates of acute GvHD appear similar between the two graft sources. Such data, however, indicate that chronic GvHD rates are higher with mobilized blood than with the use of marrow graft. There does not appear to be a survival advantage for one graft source over another, especially with longer follow-up data except in patient with high-risk leukemias where blood is the preferred source with matched-sibling donors [84–86]. It is important to recognize that myeloablative conditioning predominantly was used in most studies and HCT is a multifactorial process; hence, the results should be interpreted cautiously and cannot be generalized. However, there is firm consensus to avoid mobilized blood grafts in nonmalignant disorders and in children with malignant disorders [88].

Conditioning Regimen Intensity

Over the past several years, investigators increasingly utilize "nonmyeloablative" (NMA) or "reduced-intensity conditioning" (RIC) regimens in the allograft setting, i.e., less toxic preparative regimens that maintain reliable donor engraftment [89–91]. Successful exploitation of this approach allows patients thought to be ineligible for a full myeloablative transplant due to older age, organ function impairment, prior myeloablative transplantation, or other risk factors such as hepatitis B seropositivity, to undergo allogeneic HCT [92–96]. Conventional allografts rely upon both high-dose chemoradiation therapy for cell kill and the donor immune system to induce a graft-versus-tumor effect whereas RIC and NMA conditioning rely more on donor cell mediated immune effects and less on the cytotoxic effects [92, 93]. NMA transplants utilize regimens sufficiently immunosuppressive to facilitate donor cell engraftment, but are less toxic to both marrow and non-marrow organs (Table 57.1). These reduced-conditioning preparative regimens generally involve the use of nucleoside analogs, low-dose total body irradiation (TBI), and often antithymocyte globulin (ATG) [97–100]. One group has reported striking results when photopheresis therapy is added to the preparative regimen, although this approach has not gained wide acceptance [101]. Extracorporeal photopheresis (ECP) is a form of apheresis in which patients are given drugs such as 8-methoxypsoralen that are photoactivated. During the apheresis process, recipient blood is exposed to ultraviolet light that activates the drug. The 8-methoxypsoralen irreversibly binds covalently to both strands of the DNA of nucleated cells after photoactivation. It is theorized that the photochemically damaged T-cells, returned to the patient, do not induce GvHD, although there may be other explanations for the observed clinical efficacy of ECP. The use of ECP grafts is discussed in greater detail elsewhere in this text.

NMA approaches rely predominantly on the immunotherapeutic effect of donor cells and may be associated with higher relapse rates than those observed with ablative conditioning. To address this finding, investigators have truncated the posttransplant GvHD prophylaxis in order to induce a graft-versus-tumor effect. Donor lymphocyte infusion (DLI) is another strategy utilized to improve RIC and NMA transplant [102–104]. Lymphocytes are collected from the hematopoietic cell donor via apheresis and are infused intravenously at various intervals and doses. This modality has been demonstrated to be an effective treatment for patients who have early or impending relapse after allograft. This maneuver can provide antitumor effect and result in prolonged disease-free survival, particularly in chronic myeloid leukemia (CML) and lymphoid malignancy patients; the results of DLI are less impressive in other malignant disorders [102–106]. Although tyrosine kinase inhibitors (TKIs) have never been compared with DLI in a prospective trial for relapsed CML after allogeneic HCT, there is continued interest in utilizing TKIs posttransplant instead of DLI due to the more favorable toxicity profile [107]. Some investigators have addressed the higher relapse rates by combining an initial autotransplant followed soon thereafter by a RIC allograft [108]. Although this double transplant approach initially was initiated in lymphoid malignancies, the greater use of these so-called tandem autologous-allogeneic-transplant has been in multiple myeloma patients [109, 110]. GvHD, with or without DLI, may complicate the RIC procedure and may negate the benefit of this approach. Caution is urged for this newer modality, which will require controlled trials to define its role.

Alternative Hematopoietic Progenitor Cell Donors

HLA-Haploidentical and Cord Blood Transplants

In patients in whom an autograft is not an appropriate consideration, and in whom a HLA-sibling matched donor or HLA-MUD are not available, strategies may include the use of HLA-haploidentical donor or an UCB graft [60, 111] (see section "Selection of an Allogeneic Donor"). These approaches are considerably more complicated and are associated with significantly more supportive care needs [112]. Although a randomized trial comparing these diverse donor sources is ongoing (Blood and Marrow Transplant Clinical Trial Network protocol BMTCTN 1101), currently available comparisons rely on observational data and small phase II trials [111]. Table 57.3 illustrates the results of two concurrent, phase II BMT CTN trials that essentially demonstrated equipoise and

Table 57.3 Haploidentical marrow transplant versus unrelated double cord blood transplant with [a]reduced intensity conditioning [111]

	BMT CTN 0603	BMT CTN 0604
	Haploidentical marrow transplant	Unrelated double cord blood transplant
Total number of patients	50	50
Age	Median 48 (7–70) year	Median 58 (16–69) year
GvHD prophylaxis	Tacrolimus + mycophenolate mofetil + cyclophosphamide (days 3, 4 after transplant)	Cyclosporine + mycophenolate mofetil
Neutrophil engraftment	16 days	15 days
Platelet engraftment	24 days	43 days
Primary graft failure	1 patient	5 patients
Acute GvHD (grade II-IV) at day + 100	32%	40%
Acute GvHD (grade III-IV) at day + 100	No reported cases	21%
Chronic GvHD till 1-year	13%	25%
Progression-free survival at 1-year	48%	46%
Treatment-related mortality at 1-year	7%	24%
Non-relapsed mortality at 1-year	7%	24%
Overall survival at 6-months[b]	84%	74%
Overall survival at 1-year	62%	54%
Most common cause of death	Relapse	Relapse

[a]Cyclophosphamide, fludarabine, 200 cGy total-body irradiation
[b]Primary end point of both studies; GvHD-graft-versus-host disease; Adapted from Kekre N, Antin JH. Semin Hematol. 2016;53:98–102

provided justification to pursue the ongoing head-to-head comparison. For UCB grafts, the GvHD incidence and severity appear reduced; on the other hand, recovery of the myeloid compartment in UCB transplant recipients in adults is significantly slower than with other hematopoietic graft cell sources, i.e., a median time to neutrophil recovery >500/μL of 27 days [113]. The slow immune system recovery leads to a significant risk of opportunistic infection and treatment-related morbidity and mortality [111, 113]. Many investigations are ongoing to address these and other issues, including the infusion of two UCB units and ex vivo expansion of cells [114].

Use of HLA-haploidentical graft for transplant conversely reconstitute the recipient myeloid function rapidly but is associated with an increased risk for severe GvHD. This limitation can be attenuated by use of posttransplant cyclophosphamide (see below). Nonetheless, other problems may plague this approach, including opportunistic infection and tumor relapse [111, 115, 116]. This graft source may be more readily available and at a lower graft acquisition cost.

Supportive Care

Central Venous and Hemapheresis Catheters

Central venous catheters (CVC) are indispensable for the transplant procedure. Typically, a dedicated expert inserts a long-term, silastic, multilumen, small-bore, flexible catheter that remains in situ for many weeks to months. Such an approach requires vigilant care by a team of heath care professionals to prevent complications [117, 118]. These devices are used for: chemotherapy administration; collection of hematopoietic cells in autograft candidates undergoing the hematopoietic progenitor cell mobilization process; infusion of hematopoietic cells; supportive care management including frequent blood sampling, intravenous antibiotics, analgesics, antiemetics, blood component transfusions, electrolyte replacement, parenteral nutrition, and even performing ECP procedures [119]. Complications associated with use of central venous catheters range from pain, infection, and bleeding at the local insertion and exit sites, to bloodstream infections, venous thromboembolism, arrhythmias, mechanical obstruction, and catheter migration. Over the past 10 years, antiseptic-impregnated catheters have been developed to reduce the risk of infections in immunocompromised hosts [120]. Some centers have reported decreased rates of infectious complications with their use. Other infection control measures that have been shown to decrease catheter-associated blood stream infections include education of patients and staff regarding catheter care, good hand hygiene, aseptic technique during insertion and when accessing the device, and standard catheter dressing care guidelines [117, 121]. Additionally, catheters should be removed when they are no longer necessary. There are no data to suggest that systemic prophylactic antibiotics or routine catheter replacement decreases the risk of infectious complications.

Prevention of Nausea and Vomiting - An Important Aspect of Transplant Care

Nausea and vomiting are among the most common acute adverse events arising from high-dose chemotherapy and radiation therapy in the HCT setting [122]. Dopamine-blocking agents such as prochlorperazine previously were the mainstay in the treatment of nausea and vomiting in cancer

chemotherapy. The introduction and use of 5-HT3 receptor antagonists, i.e., serotonin antagonists, revolutionized the treatment of this malady. The first series describing this approach in HCT recipients appeared over 20 years ago [123]. Subsequently, the combination of a serotonin blocker with dexamethasone is the most frequently prescribed prophylactic agent combination to prevent emesis associated with high-dose cytotoxic therapy [124–126]. The neurokinin-1 receptor antagonists aprepitant, fosaprepitant, rolapitant, and casopitant recently have been added successfully to the armamentarium [127, 128]. “Complimentary therapy” such as acupuncture also has been used but, unlike results in the non-HCT setting, has been not found to be advantageous [129].

There are obvious differences in planning an antiemetic strategy in the HCT patient. The American Society of Clinical Oncology guidelines suggest use of the three-drug approach consisting of dexamethasone, a serotonin antagonist, and a neurokinin-1 antagonist for subjects receiving highly emetogenic chemoradiation therapy. This position is based on factors that include the recognition that TBI often is a component of therapy, the cytotoxic treatments are given for a number of consecutive days, and most HCT patients are not chemotherapy naïve [130].

Although several publications describe the use of the various 5-HT3 blockers in the HCT setting, there appear to be few differences between the various serotonin blockers as they relate to side effects, efficacy, or the combination. Palonosetron is an exception to this finding as its receptor half-life is substantially longer than the other agents and this property permits less frequent dosing [125, 126, 130]. Four important statements can be gleaned from antiemetic guidelines as such pertain to HCT. First, a serotonin blocker always should be used in conjunction with dexamethasone. Second, the minimally effective biologic (antiemetic) agent dose should be used. Third, as it appears that all current 5-HT3-blocking medications are therapeutically equivalent, the choice of agent should be determined by economic factors at each institution. Fourth, patients receiving highly emetogenic regimens may derive benefit from up front addition of neurokinin-1 receptor antagonists. Supplementary benefit from use of adjunctive agents such as dopamine blockers (haloperidol and prochlorperazine) also may be advantageous on an as needed basis.

Transfusion Support in the Hematopoietic Transplant Setting

Nearly all HCT recipients develop anemia, neutropenia, and thrombocytopenia. All cellular blood products, including red blood cells (RBCs) and platelets, contain small numbers of leukocytes. These “passenger leukocytes” are important in immunization of the recipient to HLA antigens present in the donor. Hence, transfusions from blood relatives *must* be avoided when possible for all potential allogeneic transplant candidates, and blood banks at most centers utilize a formal protocol to prevent such occurrences. Such strategy minimizes the risk of graft rejection and refractoriness to platelet transfusions [131]. The “passenger leukocytes” also may be responsible for transmission of cytomegalovirus (CMV) infection (see below) and febrile transfusion reactions (cytokine-mediated). Further, recipient immunocompromised status is a risk factor for transfusion-associated GvHD, a condition that is nearly always fatal and for which currently there is no effective therapy [132, 133]. To prevent CMV transmission and inadvertent lymphoid engraftment and proliferation, all blood components are irradiated routinely (1200–3000 cGy) prior to infusion. Irradiation of blood products usually is continued until 6–9 months after transplant, at which time, it is believed that full immunologic reconstitution has been restored. In recent years, leukocyte reduction filters have been employed before and after collection and storage of blood components, which significantly reduces the incidence of refractoriness to platelet transfusion, HLA alloimmunization, and transmission of CMV infection. These filters, however, are not able to provide adequate protection against transfusion-associated GvHD; therefore, irradiation remains the preferred method in HCT recipients [134–136].

Transplant-related nonhemolytic anemia, due, in part, to a relative endogenous erythropoietin deficiency, plagues allogeneic and autologous HCT. Red blood cell transfusions should be undertaken if Hb < 8 g/dL or Hct < 25%. A higher transfusion threshold should be considered if the patient is elderly or has symptoms of severe fatigue, headache, tachycardia, cardiac ischemia, or hypotension. In a prospective randomized trial examining administration of exogenous erythropoietin (500 U/kg per week), erythroid recovery was hastened and transfusion requirements decreased if started 1 month after allogeneic HCT. The proportion of complete correctors (i.e., Hb ≥13 g/dL) before 126 days after HCT was 8.1% in the control arm (median not reached) and 63.1% in the erythropoietin arm (median, 90 days) ($P < .001$). Hb values were higher and transfusion requirements lower ($P < .001$) in the erythropoietin arm. Of note, when erythropoietin therapy was started 28 days after transplant, only 5 patients had a Hb level higher than 11 g/dL (mean 9 g/dL), in keeping with current guidelines for initiation of erythropoietin therapy [137]. Despite these data, most transplant centers do not employ this approach.

Spontaneous bleeding, the most frequent complication of severe thrombocytopenia, is greatest at platelet counts of 20,000/μL or less. A number of investigators have advocated lower threshold levels for prophylactic transfusion in view of heightened concerns about transfusion costs and risks of infection and alloimmunization [134, 135]. Few studies have

addressed this issue prospectively and many of the trials were undertaken in leukemia patients who were not receiving HCT. Some centers administer platelet transfusions only if the count drops below 5000/μL while others maintain a 10,000 or 20,000/μL threshold. In patients with active bleeding or those scheduled to receive an invasive procedure, most physicians target a platelet count of 50,000/μL (higher in those with suspected or documented CNS hemorrhage).

Granulocyte Transfusions

For more than five decades, granulocyte transfusions have been used for treating infections in neutropenic patients; infusion of low numbers of transfused cells, however, hampered effectiveness [138]. With the advent of recombinant hematopoietic growth factors, the yield from volunteer donors given G-CSF and corticosteroids (dexamethasone) significantly increased cell dose [139]. A clinician may anticipate a rise in blood absolute neutrophil count to approximately 1,000 cells/μL if the recipient is given a collection containing 5–8 × 10^{10} granulocytes. The posttransfusion neutrophil increment may persist for more than 24 h after transfusion. This cellular product is plagued by difficulty identifying donors, high procurement costs, and short shelf life. Further, both the volunteer donor and the recipient are subject to risks in this endeavor. Donors given repeated dexamethasone doses are at increased risk for the development of subcapsular cataracts [140]. Fever, chills, and respiratory compromise due to new infiltrates or exacerbation of preexisting infiltrates may accompany a granulocyte transfusion, especially if a recipient has preexisting HLA alloantibodies [141]. The latter reaction (alloimmunization) also may be induced by the large number of lymphocytes that are contained within the product. Investigators usually screen for class I and class II HLA and anti-neutrophil antibodies before initiating a course of granulocyte transfusions in order to identify compatible donors; if not, these subjects may have subtherapeutic responses or a greater likelihood of adverse events [142]. As noted above, CMV disease also can result by transmission of this virus contained within the granulocytes. Despite these drawbacks, some clinicians advocate granulocyte transfusions for treatment of severe, progressive infections in neutropenic patients who fail to respond to antimicrobial agents and recombinant hematopoietic growth factors [143, 144]. The decisions to initiate and to continue transfusions can be difficult and a clinician carefully must consider the benefits versus the risks of granulocyte transfusions. Daily administration should be continued until control of the infection, neutrophil recovery, or development of significant toxicity. Sometimes it is unclear whether to continue this approach as patients initially may have worsening of symptoms (i.e., pulmonary, as above) due to trafficking of granulocytes to sites of infection. Such patients ultimately may experience clinical and radiologic evidence of improvement.

In an attempt to bring clarity, the National Heart, Lung and Blood Institute (NHLBI) of the United States undertook a randomized, controlled prospective, 14 center trial to evaluate the efficacy of granulocytes to treat infection in neutropenic patients. The Resolving Infection in Neutropenica with Granulocytes (RING) Study enrolled 56 patients who were given granulocytes compared to 58 control. No benefit of granulocyte transfusion was observed, but the power of the study was reduced due to low accrual [145–147]. In a post hoc secondary analysis, however, patients given higher granulocyte doses tended to have better outcomes than those receiving lower doses. These data suggest that if a clinician decides to administer granulocyte transfusions, it is important to collect and infuse at least 0.6 × 10^9 neutrophils/kg recipient weight. Newer products are being developed to help meet this target.

Cytomegalovirus (CMV) Detection and Disease

CMV is one of the leading causes of infectious morbidity and mortality in the allogeneic HCT population [148, 149]. CMV infection and disease are significantly less common in the autologous HCT setting, the exception being patients who are receiving CD34$^+$ -enriched grafts [150]. Some multiple myeloma and lymphoma CMV-seropositive patients appear to be at increased risk for CMV reactivation during autotransplant [151]. CMV infection is indicated by isolation of CMV from any body site, or the seroconversion of a patient in the absence of any clinical signs or symptoms of disease. CMV disease is defined as a positive culture from a deep tissue or clinical or histologic evidence of active viral infection. While a number of organs can be involved in the course of an allograft, CMV interstitial pneumonitis is the most severe infection.

Despite nearly eliminating CMV disease during the first 3 months after transplantation, CMV still remains an important pathogen in HCT recipients in the current era of antiviral prophylaxis and preemptive therapy [148, 149]. Transfusion-transmitted CMV infection can be reduced by the use of blood products obtained from seronegative donors. Filtered, leukocyte-depleted blood products and more recently, photochemical treatment using a combination of the psoralen amotosalen HCl and long-wavelength UV light also are extremely effective [152]. There remains, however, a 1–2% risk of transmitting CMV disease. There are no controlled studies that have investigated whether there is added benefit from the use of both seronegative and filtered blood products. CMV-seronegative patients, if possible, should receive

grafts from a CMV-seronegative donor. When using an unrelated donor, an HLA-A, -B, -C, -DR match but CMV-seropositive donor most likely will be preferred to a CMV seronegative donor of lesser match. For a lesser degree of mismatch, such as allele mismatches or mismatches on HLA-DQ, or -DP, a CMV-negative donor could be considered even if the match was worse. UCB grafts are a special situation and as such can be considered as CMV negative.

Several cohort studies show that CMV-seropositive transplant recipients and seronegative recipients of a CMV-positive graft have a persistent mortality disadvantage when compared with seronegative recipients receiving a seronegative donor graft, especially in the setting of highly immunodeficient patients, e.g., recipients of ex vivo or in vivo T-cell depletion transplants from unrelated or HLA-mismatched donors. If the only donor available for a CMV-seronegative patient is CMV seropositive, the risk of CMV transmission by the hematopoietic cell product is 20–30%; there currently are no strategies to eliminate the risk of primary CMV infection.

As discussed in the next section, immunoglobulin preparations have not been shown to improve outcome. Chemoprophylaxis with antiviral agents remains the mainstay of prevention, although this strategy results in unnecessary treatment of many patients who will not develop CMV infection or disease. Ganciclovir prophylaxis, in general, appears to be the most effective agent for CMV disease prevention but is plagued by myelotoxicity. Foscarnet clearly is efficacious but renal failure and electrolyte disturbances are significant and generally use is reserved for CMV resistance. Acyclovir and valacyclovir are not recommended to prevent CMV disease due to lesser efficacy. Because of such drawbacks, some institutions do not use prophylaxis routinely and employ preemptive strategies upon (quantitative) detection of CMV DNA in blood. The preemptive approach of treating infection, i.e., when blood titers become positive but before active disease is present, has become a standard of care at many centers. As patients may be treated in the outpatient setting when this situation occurs, valganciclovir has become an important and successful treatment option in selected patients [153].

Late onset CMV disease in high-risk populations and the immunomodulatory/immunosuppressive effects of CMV disease leading to invasive bacterial and fungal infections also contribute to the poor outcome in HCT. On the other hand, the risk of antiviral resistance in most patients remains low, except in the setting of intense immunosuppression as observed in HLA-haploidentical donor and T-cell depleted transplants. If drug resistance is suspected, genotypic testing and switching to an alternative drug is recommended as first-line approach [154]. Ganciclovir has been the mainstay of antiviral therapy but its use is associated with marrow suppression. When cytopenias are significant, foscarnet is a reasonable alternative. Viral load can be used to monitor the response to treatment. CMV gastrointestinal disease often can be treated with an antiviral drug alone. Recommendations for treating CMV pneumonia include the addition of intravenous immunoglobulin [155]. The impact of new drugs such as conventional dose maribavir has been disappointing. Few phase II, dose-ranging trials are examining higher doses of maribavir treatment of refractory or resistant CMV disease and as preemptive therapy. One investigational agent, brincidofovir (CMX001; lipid formulation of cidofovir), is an orally administered cidofovir compound conjugated to a lipid. The design is to release parent compound intracellularly and permit higher intracellular concentrations and lower plasma concentrations. One recent phase II dose-escalation study [156] showed a reduction of CMV infection or disease in patients receiving brincidofovir at doses of 200 mg/week for prophylaxis started at engraftment. The most common side effect was diarrhea in patients receiving at brincidofovir doses of 200 mg/week or higher and was dose-limiting at 200 mg twice weekly. There was no difference in renal or hematologic adverse effects between brincidofovir and placebo recipients [156]. A phase III randomized multicenter trial (SUPPRESS trial) of brincidofovir at a dose of 100 mg twice weekly is in progress using a similar trial design as the phase II trial (NCT01769170). Other agents under investigation include letermovir (AIC-246), BAY 38-4766 (nonnucleoside inhibitor), leflunomide, and artesunate (anti-malaria drug). Immunologic strategies such as adoptive transfer of CMV-specific T-cells and vaccination of the donor/recipient are labor-intensive; specialized efforts that have not yet been able to be extended on a widespread basis [148, 149]. Efforts are in development to better define the subgroups of patients who may benefit from intensified, prophylaxis strategies.

Intravenous Immunoglobulin in Hematopoietic Cell Transplant Setting

HCT recipients as well as patients with lymphoproliferative disorders have a higher incidence of infections due to secondary hypogammaglobulinemia. Intravenous immunoglobulin (IVIG) replacement has been one approach to prevent infection [157, 158]. Randomized controlled trials, however, have demonstrated conflicting results with respect to preparation type, schedule, and dose. Polyvalent immunoglobulin or hyperimmune CMV-IVIG are not superior to control for all-cause mortality. Although IVIG significantly reduced the risk for interstitial pneumonitis, the likelihood of hepatic SOS and other adverse events such as allergic reactions, fluid overload, and venous thrombosis was increased [158, 159]. Routine prophylaxis with IVIG is not supported for HCT and may be associated with impaired recovery of humoral immunity

during the period of GvHD prevention as well as thromboembolic phenomenon in hematologic malignancy patients [159]. Some investigators, however, still utilize IVIG therapy for very low serum immunoglobulin concentration within the first 3 months after HCT. Given the success in treating primary immunodeficiencies and polyneuropathies as well as in some hematologic malignancies, new initiatives in this area include the use of subcutaneous immunoglobulin as replacement therapy [160].

ABO- and Rh-Incompatible Transplants

The red blood cell (RBC) ABO system is inherited independently of the HLA system. Approximately 40% of all allogeneic HCT are performed successfully across either major or minor ABO-incompatible barriers between donor and recipient; these HCTs are not usually associated with an increase in grade II–IV acute GvHD or graft rejection [161, 162]. RBC antigen types may present obstacles if the donor has antibodies (isoagglutinins) against recipient RBCs (minor ABO mismatch), or if the recipient has antibodies against RBCs to donor (major ABO mismatch) antigens. Additionally, if there is a state of incompatibility in the Rh and other red cell antigen systems, significant adverse events may result [161–164]. In contrast to routine blood transfusion, the risk of hemolysis occurs not only at the time of infusion, but also may manifest weeks later if viable lymphocytes contained within the graft produce isohemagglutinins directed against recipient RBC antigens. This condition is termed the so-called *passenger lymphocyte syndrome* (see below) [161, 162].

Major ABO incompatibility presents a clinically significant risk of hemolysis against the RBCs in the graft as a result of host isohemagglutinins directed against donor cells [162]. Peripheral blood HCT grafts are collected via apheresis and contain low RBC numbers. As a result, hemolysis risk is much lower with this graft source compared to a marrow graft that contains a larger volume of RBCs. The risk of clinically relevant hemolysis is reduced by removal of RBCs from the graft using any of a variety of separation or sedimentation techniques, or reduction of isoagglutinin titer from the recipient by an apheresis approach. Isohemagglutinin titers can be depleted by large volume plasma exchange performed on the day of graft infusion and on additional days for patients with higher titers. Major ABO-incompatible HCT recipients, however, may experience delayed RBC recovery and prolonged transfusion up to 1 year later after transplantation because of immune-mediated delayed erythropoiesis with reticulocytopenia as well as slightly slower neutrophil recovery. Various strategies, including extracorpeal immunoadsorption columns, DLI, and rituximab, have been used [162] (Table 57.4).

Table 57.4 Immunologic consequences and standard prevention methods to avoid complication in major and minor ABO incompatible transplants

ABO major mismatch		
Consequences	*Etiology*	*Standard prevention methods*
Acute hemolysis	Infusion-incompatible RBCs	• Monitor for acute hemolytic reaction
Delayed neutrophil and platelet engraftment	Expression ABO antigens on granulocytes and platelets	• If recipient anti-donor titer ≥1:32 and graft contains >20 mL of RBC, deplete graft of RBC, or deplete isoagglutinin in recipient
Delayed RBC engraftment	Host anti-donor isoagglutinins	• If ≥1:32 but graft with <20 mL RBC, OR ≤1:16, infuse graft without modification
Pure RBC aplasia	Persistence of anti-donor isoagglutinins	
ABO minor mismatch		
Consequences	*Etiology*	*Standard prevention methods*
Acute hemolysis	Donor plasma with high isoagglutinin titers	• Monitor for delayed hemolytic transfusion reaction
		• If donor anti-recipient titer ≥ 1:256, plasma depletion of graft
		• If donor anti-recipient titer ≤1:128, infuse without modification
Delayed hemolytic (DAT positive) anemia	Passenger lymphocytes producing anti-host isoagglutinins	

Patients who undergo HCT using minor ABO-incompatible grafts, in contrast to major ABO-incompatible transplants, face two risks: immediate hemolysis from the infusion of incompatible plasma (that can be avoided by plasma removal prior to infusion), as well as delayed, but life-threatening hemolysis mediated by viable donor lymphocytes, i.e., "passenger lymphocytes" [162]. These cells may transiently produce sufficient concentrations of isohemagglutinins which result in potentially life-threatening hemolysis of recipient RBCs 7–14 days after transplantation, sometimes requiring hemodialysis. This situation is distinguished from transplantation-associated thrombotic microangiopathy by a positive direct antiglobulin test (DAT) (Table 57.4). Recipients at greatest risk are those who receive mobilized blood rather than marrow grafts (due to greater numbers of blood T cells infused in the graft), blood group A recipients whose donors have hemolytic anti-A isohemagglutinins in high titers, and omission of the anti-lymphocyte agents methotrexate or mycophenolate mofetil (MMF) as part of GvHD prophylaxis. Some centers advocate an aggressive preventative approach to all

ABO-incompatible transplants using RBC exchange, while others utilize aggressive interventions only for symptomatic management [162]. It is of note that donor isohemagglutinins are not produced in patients undergoing ABO-incompatible UCB transplants, an advantage of this graft source [165].

Non-ABO red cell alloantibodies can develop after HCT [161–164]. Rh incompatibility is usually not a major concern except if an Rh negative recipient is alloimmunized and receives an Rh positive graft. In that case, acute hemolysis may occur upon infusion of the graft. Transfusion of Rh positive (D positive) RBCs into Rh negative (D negative) persons frequently can result in alloimmunization and the development of anti-D antibodies. In the immunosuppressed Rh negative subject given a hematopoietic graft from an Rh positive donor, however, the likelihood of such an occurrence is low, i.e., < 10%; the higher rates are recorded in subjects given RIC or nonmyeloablative conditioning compared to myeloablative conditioning recipients [163]. In contrast, Rh positive patients who receive a graft from an Rh negative donor should be given Rh negative (D negative) blood products in the posttransplant period due to the risk of developing anti-D antibodies. To decrease the risk of alloimmunization, the use of RhIg (Rh immune globulin) is a reasonable and safe alternative [166]. In the setting of severe thrombocytopenia (or neutropenia) in which intramuscular injection of Rh Ig (RhoGam®, Kedrion Biopharma, Ft. Lee, NJ) may be associated with significant risks, IV RhIg (WinRho®, Cangene, Winnipeg, Canada) should be considered. In the latter setting, a dose of WinRho® 1500 IU or 300 mcg IV will neutralize 15 mL of Rh positive RBC. The practitioner should be mindful that use of WinRho may be associated with severe hemolysis [167]. Additionally, infusion of platelet concentrates for transfusion and granulocytes transfusions may contain RBC and consultation with a blood bank colleague or expert in transfusion medicine should be undertaken.

Prevention and Treatment of Mucositis

Mucositis is a frequent and significant complication in patients undergoing HCT, contributing to local as well as systemic complications [168–170]. Mucositis of the oral cavity causes pain, barrier disruption leading to an increased risk of infection, and sometimes septicemia (often with *Streptococcus* sp.), especially in the neutropenic host. From the patient's point of view, mucositis may be the most debilitating complication of transplant. Reactivation of HSV can exacerbate preparative regimen-induced mucositis and HSV prophylaxis is important. Mucositis increases the financial cost of care and it may extend the length of hospitalization and even increase mortality; a retrospective study showed a fourfold increase in mortality in subjects with severe oral mucositis [171]. A thorough evaluation of the patient's oral cavity by a dental specialist is essential and should include imaging such as panorex before initiating the preparative regimen. A dental cleaning and removal of diseased teeth is strongly recommended. Monitoring of oral mucositis during HCT is difficult due to lack of a reliable, objective, and valid scale, especially given the high subjectivity of pain severity that significantly influences the evaluation. In an effort to improve research in this area, the Oral Mucositis Index (OMI) assesses the severity of oral mucositis in terms of erythema, ulceration, atrophy, and edema (each graded on a scale of 0–3, where 0 = none and 3 = severe). This approach is internally consistent with high test–retest and inter-rater reliability and exhibits strong evidence of construct validity but is cumbersome to apply in routine practice [172]. The most commonly used, the National Cancer Institute Common Terminology Criteria for Adverse Events (NCI CTCAE), grades the severity of mucositis on a scale from 1 to 5, (Table 57.5) based upon symptomatology, clinical findings, and interventions required [173]. During HCT, it is permissible to allow removable dentures to remain in place while eating but otherwise this appliance should be removed and placed in appropriate antimicrobial/antifungal solutions during storage. Cryotherapy prophylaxis (ice chips or iced medications) historically has been a useful adjunct by decreasing blood flow to the mucus membranes but is useful only with chemotherapy agents that have a very short half-life, i.e., single agent melphalan. Amifostine is a parenterally administered organic thiophosphate prodrug which is hydrolyzed in vivo by alkaline phosphatase to the active cytoprotective thiol metabolite. This agent has been shown not only to reduce the incidence of mucositis in HCT patients but also decrease the use of parenteral nutrition and narcotic use directly related to degree of mucositis [174]. Anticholinergics such as propantheline are thought by some to be useful as these agents may decrease the excretion of parenterally administered drugs into saliva. Medicated rinses also have been used but alteration of pH by some agents such as hydrogen peroxide

Table 57.5 Oral mucositis grading: National Cancer Institute common terminology criteria for adverse events

	Grade 1	Grade 2	Grade 3	Grade 4	Grade 5
Oral mucositis	Asymptomatic or mild symptoms; intervention not indicated	Moderate pain; not interfering with oral intake; modified diet indicated	Severe pain; interfering with oral intake	Life-threatening consequences; urgent intervention indicated	Death

Reproduced from: Common Terminology Criteria for Adverse Events (CTCAE), Version 4.0, June 2010, National Institutes of Health, National Cancer Institute. Available at: http://evs.nci.nih.gov/ftp1/CTCAE/CTCAE_4.03_2010-06-14_QuickReference_5x7.pdf (Accessed June 8, 2017)

can retard healing of injured areas. Topical analgesics have been utilized including morphine and ketamine rinses but both need to be used cautiously [175, 176]. Other potentially beneficial agents include povidone–iodine, chlorhexidine, and topical granulocyte-macrophage colony-stimulating factor (GM-CSF). Nystatin and clotrimazole troches are useful to prevent oral fungal infections; topical tetracyclines in combination with diphenhydramine and glucocorticoids may avert bacterial infections in the oral cavity. Laser therapy has been used for both prevention as well as treatment of oral mucositis [177, 178].

Recombinant human keratinocyte growth factor (KGF; palifermin), a heparin-binding member of the fibroblast growth factor family, represents another effective approach for management of acute reactions in oral, gastrointestinal, and cutaneous epithelia after radiation exposure [179, 180]. Animal and human models indicated that this agent causes thickening of the nonkeratinocyte layers of the oral mucosa and digestive tract [181]. For conditioning regimens that utilize TBI during autologous HCT, palifermin has been somewhat effective in reducing the duration of mucositis of parenteral nutrition and increasing quality of life scores [179, 180]. The effect on hematopoietic cell engraftment as well as GvHD and overall morbidity is yet to be fully defined. Newer therapeutic approaches still in early development include gamma-D-glutamyl-1-tryptophan and D-methionine [182]. The published guidelines outline the foundation for management of mucositis related to chemoradiotherapy management [183, 184].

Sinusoidal Obstructive Syndrome

Significant hepatic injury in the course of a myeloablative HCT may affect as many as half of transplant recipients. Etiologies include infections, high-dose chemoradiation therapy, malignancy, antibiotics, parenteral alimentation, immunosuppressive agents, hematopoietic growth factors, and GvHD; overlapping clinical and laboratory features are present with many of these conditions [185–187]. Hepatic sinusoidal obstructive syndrome (SOS), formerly referred to as hepatic veno-occlusive disease (VOD), is an often fatal syndrome characterized by jaundice, fluid retention, and painful hepatomegaly. This condition may range in severity from mild and reversible to one associated with fatal multi-organ failure (MOF), hepato-renal syndrome, and mortality. The diagnostic criteria [186–188] have limitations (Table 57.6), often leading to misdiagnosis and at times, late diagnosis. In adults, the so-called Baltimore criteria [187] may be preferred over the so-called Seattle criteria [186]. Neither criterion considers the case of late-onset SOS typically appearing more than 21 days ("late-onset") after HCT [189]. Few studies highlight the role of monitoring specific

Table 57.6 Routinely employed diagnostic SOS criterias and their limitations in HCT.

HCT-related SOS criteria		Limitations
Modified Seattle criteria [186] (1984)	Presence of at least two of the following within 20 days of HCT:	• Cannot reliably predict natural course of disease
	1. Serum total bilirubin concentration >2 mg/dL	• Cannot realibily predict severity of disease
	2. Hepatomegaly or right upper quadrant pain	• Do not consider "late-onset" disease
	3. Sudden weight gain (>2% of baseline body weight) due to fluid accumulation	• Additional testing may be required for correct diagnosis
Baltimore criteria [187] (1987)	Serum bilirubin >2 mg/dL within 21 days of HCT plus at least two of the following	
	1. Hepatomegaly	
	2. Ascites	
	3. Weight gain >5% from pre-HCT weight	

serum biomarkers, i.e., procollagen type III, protein C, plasminogen activator inhibitor type 1, with the aim to diagnose and classify the severity of SOS [190–193]. At present, the presence of multi-organ failure (MOF) in which the kidneys, lungs, or central nervous system are injured appear to be the best clinical marker of severe SOS [194]. The area and role of imaging for diagnosis [195] remain of interest and active investigation. Fortunately, in most patients (75–80%), SOS progressively resolves over a 2–3-week period. Nevertheless, severe SOS is a potentially life-threatening complication associated with a high mortality rate (> 80%), and methods or models to prevent or attenuate poor prognostic disease are warranted. The rate of rise in serum bilirubin (especially in adults) and weight gain may be more rapid for patients whose illness is likely to be severe versus those who are likely to have self-limited disease.

The pathogenesis of SOS is believed to reside at the level of venous hepatic endothelial cell. Histologically, development of hepatic SOS demonstrates damaged sinusoidal endothelial cells with venular obstruction and centrilobular hemorrhagic necrosis in zone 3 of the liver acinus. Sinusoidal obstruction is prominent and has led to use of the term SOS, although this designation has not been adopted universally. Continued injury causes deposition of collagen, sclerosis and fibrosis of hepatic venules and sinusoidal occlusion which ultimately lead to liver failure and death [196–199].

Hepatic SOS is much more common in allogeneic rather than autologous HCT recipients. The current incidence of SOS varies between studies and is approximately 10–15% after allogeneic HCT using myeloablative conditioning regimens and is <5% after RIC or autologous HCT [196, 200,

201]. Risk factors for the development of hepatic SOS include a history of previous hepatocellular disease, evidence of active liver injury (elevated AST levels) at time of initiating the preparative regimen, use of busulfan-containing preparative regimens, exposure to the monoclonal antibody gemtuzumab ozogamicin, children age < 7 years, older age adults, female gender, donor–recipient HLA disparity, advanced malignancy, prior abdominal radiation, second myeloablative conditioning transplant, reduced corrected pulmonary diffusion capacity (DLCO), recent fungal infection, and poor performance status at start of transplant (Table 57.7) [185, 186, 188, 198, 200–206].

A number of interventions have been associated with variable success as prophylaxis and as therapy. These strategies include use of tissue plasminogen activator, antithrombin III and prostaglandin E1, low-dose heparin, and low molecular weight heparins as well as non-pharmacologic interventions (transjugular intrahepatic portosystemic shunts [TIPS], and charcoal hemofiltration). Ursodeoxycholic acid, a hydrophilic nonhepatotoxic bile acid, appears to be useful as an agent that if begun many weeks in advance of HCT can prevent hepatic SOS and other hepatic complications of HCT [207, 208]. One of the most promising agents appears to be defibrotide, a single-stranded polydeoxyribonucleotide that possesses anti-ischemic, anti-thrombotic, and thrombolytic activity but without significant anticoagulant effects [201, 202, 209, 210]. Multiple studies have demonstrated 30–60% complete remission rates with defibrotide, even among patients with severe SOS and MOF. Defibrotide has been approved by the US Food and Drug Administration and European Medicines Agency for the treatment of adults and children affected by SOS with renal or pulmonary dysfunction after HCT. Recommendations regarding use of defibrotide in the prophylactic setting await the results of randomized, prospective trials. Guidelines from the British Society for Blood and Marrow Transplantation [201], however, recommend its use in children (IA) and adults (IIA) at high risk of SOS: preexisting hepatic disease, second myeloablative conditioning HCT, allogeneic HCT for leukemia beyond second relapse, conditioning with busulfan (Bu)-containing regimens, prior treatment with gemtuzumab ozogamicin, or a diagnosis of primary hemophagocytic lymphohistiocytosis, adrenoleucodystrophy or osteopetrosis. The use of IV busulfan and dose adjustment based on plasma drug concentration profoundly have decreased the incidence of SOS.

Table 57.7 Potentially modifiable risk factors associated with SOS in HCT

HLA disparity
Higher dose of gemtuzumab ozogamicin prior to HCT (Newer FDA approved dose may be safer)
High serum ferritin concentrations
Myeloablative regimen in subjects who have pre-existing liver disease
Avoidance of hepatotoxic drugs
Avoidance of HCT in subjects who have a corrected DLCO <70%
Use of certain myeloablative regimen (Bu/Cy>Bu/Flu)
Myeloablative TBI containing regimens (single dose > fractionated doses)
Oral busulfan (as opposed to intravenous busulfan)
Drug sequence of conditioning regimen (Cy/Bu instead of Bu/Cy)
Sirolimus plus methotrexate as GvHD prophylaxis in myeloablative setting

Bu/Cy busulfan/cyclophosphamide, *Bu/Flu* busulfan/fludarabine, *Cy/Bu* cyclophosphamide/busulfan, *GvHD* graft-versus-host disease

Transplant-Associated Thrombotic Microangiopathy: A Distinct Entity

Transplant-associated thrombotic microangiopathy (TA-TMA) refers to inflammatory and thrombotic diseases of the microvasculature characterized by Coombs-negative hemolytic anemia, presence of schistocytes and thrombocytopenia on peripheral blood smear, elevated serum LDH, decreased serum haptoglobulin concentrations, and evidence of organ damage, particularly acute renal failure [211–215] (Table 57.8). Increasing evidence suggests that TA-TMA is a multifactorial disorder that is distinct from thrombotic thrombocytopenic purpura (TTP), and likely represents the final common pathway of a number of endothelial cell insults [214–216]. This syndrome is estimated to occur in up to 10–20% of patients who undergo allogeneic HCT [211–216]. True estimates of the rate at which TA-TMA occurs in the allogeneic HCT setting, however, are difficult to make because of inconsistencies in the diagnostic criteria applied and because of the overlap of clinical signs and laboratory abnormalities with posttransplant complications that mimic TA-TMA. In an attempt to standardize the diagnosis, two groups developed separate guidelines [217, 218]. A validation study published by Cho and coworkers, [216] noted limitations and expanded the guidelines to include the concept of “probable-TMA,” which does not require renal or neurologic findings. This condition is more frequently seen when calcineurin inhibitors are combined with sirolimus and in patients with acute GvHD.

TA-TMA presents diagnostic challenges because the syndrome may not clearly fall into one of the categories of two major conditions similar in scope and presentation: atypical hemolytic uremic syndrome (aHUS) and TTP. In addition, complications of the transplant itself, including infection, GvHD, and disseminated intravascular coagulation, as well as the side effects of immunosuppressive drugs, can mimic TMA. To highlight the diagnostic challenge of TA-TMA, Inamoto and coworkers reported that 92% of transplant recipients undergoing colonoscopy for severe diarrhea thought to be secondary to GvHD had histologic evidence of TA-TMA, while only 30% had concomitant histologic evidence of

Table 57.8 Different diagnostic criterias for TA-TMA

Blood and Marrow Transplant Clinical Trials Network (BMT CTN) toxicity committee consensus definition for TMA [217]
1. RBC fragmentation and x 2 schistocytes per high-power field on peripheral blood film
2. Concurrent increased serum LDH above institutional baseline
3. Concurrent renal and/or neurologic dysfunction without other explanations
4. Negative direct and indirect Coombs test results
International Working Group Definition for TMA [218]
All of the following are present:
1. Increased percentage (44%) of schistocytes in the blood
2. De novo, prolonged or progressive thrombocytopenia (platelet count less than 5×10^9/L or a 50% or greater decrease from previous counts)
3. Sudden and persistent increase in serum LDH
4. Decrease in hemoglobin concentration or increased red blood cell transfusion requirement
5. Decrease in serum haptoglobin concentration
Probable TMA as defined by validation study by Cho and coworkers [216]
1. RBC fragmentation and × 2 schistocytes per high-power field on peripheral blood film
2. Increase in serum LDH
3. Thrombocytopenia: $\leq 50 \times 10^9$/L or a $\geq$ 50% decrease in platelet count
4. Decrease in hemoglobin
5. Decrease in serum haptoglobin concentration
6. Negative Coombs test results
7. Absence of coagulopathy

GvHD [219]. The low blood concentration of ADAMTS13 (a disintegrin and metalloproteinase with a thrombospondin type 1 motif, member 13) helps establish the diagnosis of other conditions including non-transplant associated TTP/HUS. Activity above 5–10% is found in most patients with TA-TMA. The pathophysiology of TA-TMA is poorly understood but includes loss of endothelial cell integrity induced by intensive conditioning regimens, immunosuppressive therapy, irradiation, infections, and GvHD. Interestingly, a recent study suggested that the presence of the HLA-DRB1*11 antigen may contribute to the development of TA-TMA [220]. Unfortunately, the treatment outcomes of TA-TMA remain suboptimal. Therapy should include cessation or dose reduction of calcineurin inhibitors and sirolimus. While often utilized, plasma exchange should be discouraged as morbidity and mortality are quite high due to the introduction of infection and complications of venous access devices. The anticoagulant defibrotide, the humanized monoclonal antibody eculizumab directed against complement protein C5, and rituximab have been used with some success [211–215, 221, 222]. Future research should emphasize the discovery of "early" markers as the successful treatment of TA-TMA may depend on early initiation of therapy.

Prevention and Treatment of Hemorrhagic Cystitis: An Emerging Concern

Hemorrhagic cystitis can be seen early or late in the HCT setting, and results from bladder mucosal injury as a result of chemotherapy, radiation, or viral infection [223–225]. The condition rarely is fatal, but the morbidity can be considerable and can be associated with infection, multi-system organ failure and ultimately death; as a result, some centers employ very aggressive strategies or prophylaxis. Patients may develop microscopic or gross hematuria, dysuria, and bladder pain, usually within 3 weeks of HCT, or as late as several months after transplant. This condition may lead to major urinary obstruction, sepsis, renal failure, or the need to undertake surgical or chemical bladder cauterization. Some high-dose chemotherapeutic agents, eliminated, in part, via the urinary tract such as cyclophosphamide, thiotepa, busulfan, ifosphamide, and etoposide, can initiate injury that is exacerbated by the development of neutropenia, thrombocytopenia, or the combination. Most investigators recommend platelet transfusions to keep the platelet count at least 50,000/μL once the injury occurs. Reactivation of Bk virus is the other major cause of hemorrhagic cystitis, although CMV and other virus infections have been reported [224]. Asymptomatic Bk virus infection occurs during childhood and 90% of adults are seropositive; reactivation risk factors and associated hemorrhagic cystitis include myeloablative conditioning, allogeneic HCT, HLA-haploidentical transplants, use of unrelated donors, and development of GvHD [224, 225]. Interestingly, use of busulfan was shown to be associated with significantly greater Bk virus-associated hemorrhagic cystitis compared to myeloablative HLA-haploidentical transplant using a TBI based regimen [226]. The finding of Bk virus in the urine usually precedes the occurrence of hematuria. Urine polymerase chain reaction (PCR) usually can detect the virus but if clinical suspicion is high and urine testing negative, cystoscopy and biopsy of the bladder epithelium should be undertaken. If indicated, immunosuppressive medications should be decreased. Ciprofloxacin therapy, given for prolonged periods, often is the treatment of choice. Cidofovir given either systemically or via the intravesical route has been effective [227, 228]. Aggressive intravenous hydration (100–150 mL/m^2/h), liberal use of diuretics, and frequent voiding have been reported as effective preventative measures. Intravenous mesna therapy, a uroprotectant with a free sulfhydryl group, inactivates acrolein and the active alkylating metabolites of cyclophosphamide and ifosphamide and usually is effective as prevention.

Treatments for hemorrhagic cystitis range from simple measures to invasive approaches. Insertion of a three-way Foley urinary bladder catheter with saline lavage is the mainstay

of treatment. A variety of other approaches have been used in patients who have refractory bleeding and include ganciclovir, instillation of alum (an astringent), prostaglandins, hyperbaric oxygen, epsilon-aminocaproic acid, conjugated estrogen formalin, hyaluronic acid, recombinant human GM-CSF, and palifermin [229–232]. Finally, one group has reported that IV MSC therapy may be useful [233].

Graft Failure and Rejection

HCT requires infusion of an adequate hematopoietic progenitor cell dose after administering high-dose chemoradiation therapy. Quantifying $CD34^+$ cell surface antigen expression has been demonstrated to be a reliable predictor of sustained hematopoietic recovery after transplant. In the autologous HCT setting, data suggest a dose–response relationship between the number of $CD34^+$ cells infused and engraftment kinetics [234]. It is estimated that delayed hematopoietic engraftment is observed in 10–20% of non-Hodgkin lymphoma patients undergoing autotransplant [235]. Although multiple studies have attempted to define the required $CD34^+$ cell dosage for a successful HCT, the results are not uniform and many physicians consider a cell dose of $2.0–2.5 \times 10^6$ $CD34^+$ cells/kg of body weight as a minimum for achieving hematopoietic reconstitution (usually recovery of neutrophils) within a reasonable timeframe. Infusion of a cell dose about 5.0×10^6 $CD34^+$ cells/kg of body weight, however, seems to be optimal. Enhanced engraftment kinetics translate into fewer RBC and platelet transfusions, diminished antibiotic usage, shorter hospital stay, and decreased overall costs.

In some patients, despite what are considered effective stimuli, an insufficient number of $CD34^+$ cells are mobilized. This group of patients may otherwise be acceptable candidates for autotransplant, but are precluded from the procedure due to insufficient cells for infusion. If autologous HCT is attempted, these subjects are at significantly increased risk for infections, bleeding, and other complications associated with slow or incomplete engraftment. This "hard-to-mobilize" group usually can be identified by a number of factors, including exposure to hematopoietic stem cell toxins such as fludarabine, nitrogen mustard, melphalan, BCNU (carmustine), extensive prior chemotherapy or radiation therapy, advanced age, or tumorous involvement of bone marrow [236, 237].

Plerixafor (Mozobil™, Genzyme Corp.), in combination with hematopoietic growth factors, has been shown to be effective for mobilization of hematopoietic progenitor cells in NHL and multiple myeloma patients [236, 237]. This agent acts as an antagonist for the CXCR4 chemokine receptor and blocks homing of hematopoietic stem cells to the bone marrow stroma. In combination with hematopoietic growth factors, plerixafor increases $CD34^+$ cell collection, decreases the number of apheresis sessions, and allows more patients to proceed to autologous HCT. Side effects are minimal and include gastrointestinal distress, injection site reactions, headache, and fatigue. Studies using other mobilizing agents in patients and use of plerixafor in volunteer hematopoietic cell donors for allogeneic HCT are ongoing [238].

Failure of donor cells to fully engraft after myeloablative therapy in the allogeneic transplant setting may reflect infusion of insufficient numbers of donor hematopoietic progenitor cells, splenomegaly, a damaged marrow microenvironment, anti-HLA antibodies, viral infection, and inadequate GvHD prophylaxis [239–241]. This problem is more common with use of UCB grafts, haploidentical grafts, T-cell depletion of the graft, increased donor:recipient HLA disparity, use of RIC, insufficient preparative regimen immunosuppression, or infection due to CMV, HHV6, HHV8, or parvovirus [242–245].

Whatever the etiology, graft failure or rejection is a life-threatening process requiring immediate intervention [245]. Various solutions include withdrawal of potentially marrow-suppressive agents such as antibiotics, administration of recombinant hematopoietic growth factors, infusions of additional hematopoietic progenitor cells (if available), and in the case of a failed allograft, the decision to perform a second transplant [246]. The outcome for patients experiencing this problem generally is poor and has led some investigators to utilize other grafts for salvage [246–252]. Early recognition and institution of back up grafts can rescue some patients [253].

Acute Graft-versus-Host Disease

Allogeneic HCT in humans is hampered by the development of both acute and chronic GvHD resulting in significant morbidity and mortality, even when grafts from HLA-identical (10 of 10 matched) siblings are used [43, 254–256]. This syndrome results when genotypically disparate, alloreactive donor-derived T-cells recognize and react to host histoincompatible antigens in a host incapable of rejecting such cells. Other mechanisms, however, have been implicated including massive disequilibrium in tissue specific immunoregulation [257] and imbalance in the $CD4^+$ $CD25^+$ regulatory T-cells (T_{regs}); these cells are generated in the thymus and are thought to play a key role in the maintenance of immunologic self-tolerance. In preclinical studies, these Tregs have been shown to prevent GvHD by suppressing alloreactive donor T-cells, while preserving graft-versus-leukemia (GvL) effect [258]. Patients noted to have a higher relative frequency of Tregs after allo-HCT had a lower rate and severity of GvHD, lower rate of nonrelapse mortality, and equivalent relapse mortality [259].

When donor and recipient are not HLA identical, differing by even a single antigen, severe GvHD can ensue with an increase in mortality [260]. If a transplant is performed between HLA-identical siblings, GvHD appears to be mediated via the minor histocompatibility antigens. Both animal and human studies have demonstrated that B cells are involved in the immunopathophysiology of acute and chronic GvHD [261, 262]. Some preliminary clinical trials have shown that B-cell depletion using the monoclonal antibody rituximab may result in a beneficial effect on both acute and chronic GvHD [262, 263]. These data have implications for the clinical management of patients undergoing allogeneic HCT.

Acute GvHD is a consequence of complex interactions between donor and host innate and adaptive immune responses and multiple inflammatory molecules and cell types are involved [261]. Cytotoxic chemo- or chemoradiation therapy that comprises the conditioning or preparative regimen damages recipient tissues such as skin, gastrointestinal tract mucosa, liver, and other organs. This damage triggers release of multiple inflammatory cytokines and stimulates the preexisting antigen disparities between host and graft tissue. Other factors involve the cellular sensors that detect these triggers and present alloantigens. The process is escalated via mediators such as various T-cell subsets including naive, memory, regulatory, Th17, and natural killer T-cells that interact with effectors and amplifiers; this interaction results in damage of target organs.

A lack of understanding of the molecular mechanisms involved in the initiation phase of GvHD has been one obstacle in addressing effective prophylaxis. Recently, important mechanisms have been identified regarding the induction of GvHD through the interplay of microbial-associated molecules and innate immunity such as Toll-like receptors. This new information has opened a new area, i.e., microbiome, for future therapeutic approaches [264–266].

GvHD traditionally has been divided into acute and chronic GvHD phases, predominantly based on time of onset and clinical presentation. Acute GvHD occurs within the first 100 days after allograft, usually within the first 2–6 weeks. A hyperacute, fulminant form can occur within the first week of transplant but is rare unless patients do not receive GvHD prophylaxis [267]. The target organs for this syndrome usually are the skin, gastrointestinal mucosa, and liver; sometimes the lung, lymph nodes, and bone marrow become involved. Formerly, if the onset of a syndrome with clinical manifestations of acute GvHD occurred beyond 100 days after HCT, it was called chronic GvHD. In 2009, the National Institutes of Health (NIH) established that clinical manifestations, not time after transplantation, distinguish chronic GvHD from late onset acute GvHD [268]. The latter condition appears to be more common in patients who have undergone RIC or nonmyeloablative allogeneic HCT and it also includes persistent, recurrent, or late-onset acute GvHD. While acute GvHD is a clinical syndrome that can be diagnosed in the absence of a tissue diagnosis, it is preferred to obtain biopsies of affected tissues to firmly secure the diagnosis.

Several investigators have used a panel of serum biomarker proteins (interleukin-2-receptor-alpha, tumor-necrosis-factor-receptor-1, interleukin-8, and hepatocyte growth factor) to confirm the diagnosis of acute GvHD at onset of clinical symptoms [269–273]. This testing may provide prognostic information independent of GvHD severity and is an area of active investigation

Risk factors for developing acute GvHD include donor–recipient genetic disparity, full myeloablative compared to RIC regimens, advanced patient and donor age, gender mismatch (female donor to male recipient), use of T-replete bone marrow and blood grafts (blood > marrow grafts) compared to T cell-deplete grafts, or UCB units and prior splenectomy [274]. Another factor contributing to the increased frequency of acute GvHD is use of donor lymphocyte infusion (DLI), often administered for patients in relapse after allogeneic HCT as discussed earlier [102–107].

Acute GvHD traditionally has been quantified as grade I, II, III, or IV using clinical criteria, although the reproducibility for diagnosis and grading is problematic (Table 57.9) [275, 276]. Several groups have offered modifications of this grading system to improve the correlation between extent of GvHD and outcome; these systems are not in widespread use [277–279]. Clinically significant acute GvHD (grade II–IV) usually occurs in 10–50% of HLA-identical sibling-matched recipients compared to rates as high as 70–90% in unrelated HLA-matched and related HLA-haploidentical transplants [274–279]. The usual strategy is prophylaxis with drugs to partially suppress the graft by preventing or attenuating GvHD while maintaining an allogeneic or graft-versus-tumor effect. Immunosuppression with combinations of agents such as cyclosporine, tacrolimus, methotrexate, and corticosteroids are more effective at reducing the incidence and severity of acute GvHD and improving overall survival (OS) compared to single agents [280–282]. The regimens used most frequently include either cyclosporine or tacrolimus, in combination with methotrexate, the latter agent given usually for short periods [281, 282]. A recent survey involving 79 centers from the European group for Blood and Marrow Transplantation (EBMT) highlighted that there is no currently agreed upon standard regimen, and clinical practice varies by institution [283].

A number of newer agents are being used to prevent development of acute GvHD [280–284]. Sirolimus, in combination with other agents such as tacrolimus, appears to be an interesting and potentially more effective drug [284]. Sirolimus, however, is associated with significant adverse drug interactions and is associated with higher risk of hepatic SOS [206]. A recent, open label phase 3 study assessed the

Table 57.9 Staging of Acute GvHD

Organ	Skin	Liver	Gastrointestinal
Individual organ staging			
Stage	**Body Surface Area**	**Bilirubin (mg/dL)**	**Diarrhea (mL/day)**
1	Rash <25%	2–2.9	500–1000 or biopsy-proven upper GI involvement
2	Rash 25–50%	3–6	1000–1500
3	Rash >50%	6.1–15	1500–2000
4	Generalized erythroderma with bullae	> 15	> 2000 or severe abdominal pain with or without ileus
Revised Glucksberg grading [275]			
I	Stage 1–2	None	None
II	Stage 3 or	Stage 1 or	Stage 1
III	–	Stage 2–3 or	Stage 2–4
IV	Stage 4 or	Stage 4	–
International bone marrow transplant registry grading [276]			
A	Stage 1	None	None
B	Stage 2	Stage 1 or 2	Stage 1 or 2
C	Stage 3	Stage 3	Stage 3
D	Stage 4	Stage 4	Stage 4

For bilirubin in μmol/L: Stage 1 = 34–50, stage 2 = 51–102, stage 3 = 103–255, stage 4 > 255

combination of tacrolimus and sirolimus compared to methotrexate and tacrolimus after HLA matched related donor blood cell transplantation [285]. The combination of tacrolimus and sirolimus had equivalent efficacy with regard to GvHD prevention but showed marked improvement in hematopoietic engraftment over the other combination. Use of the sirolimus-containing regimen did not impact mortality. Mycophenolate mofetil (MMF), an antibiotic with strong immunosuppressant properties, also has become a commonly used drug in GvHD prophylaxis [286, 287]. A less common approach to prevent acute GvHD is ex vivo T cell depletion (TCD) of the graft. This maneuver can be performed by a variety of means including physical separation, density gradient, flow cytometry, lectin depletion, cytotoxic agents, monoclonal antibodies, immunoconjugates, and magnetic beads [288]. Although TCD reduces the incidence of acute GvHD, to date this strategy is associated with an increase in engraftment failure, tumor relapse, opportunistic infection, and secondary cancers. T-cell depletion trials have not demonstrated an improvement in OS [289]. Anti-T-cell globulin (antithymocyte globulin) when used in the preparative regimen has been shown to reduce the incidence of acute GvHD without significant impact on survival [290–292]. Low-dose alemtuzumab combined with cyclosporine also has shown some early promise [293]. High-dose cyclophosphamide (50 mg/kg/day IV on the third and fourth days after transplant) is given as an innovative approach and has been successful in reducing the incidence of acute GvHD in the haploidentical setting [294, 295]. Pre-clinical models demonstrate that the cyclophosphamide eradicates the proliferating alloreactive T cells. Although this strategy has been employed successfully in the HLA-matched setting, more data are required before recommending incorporation into routine practice [296].

The treatment of acute GvHD has been summarized in recommendations by expert panels in recent years [283, 297]. For patients who have predominantly GvHD confined to the skin that is not fully responsive to corticosteroids, ECP may be an effective, less toxic therapeutic approach [298, 299]. More intensive treatment, however, is necessary for grade II–IV acute GvHD. The initial approach consists of continuing the original immunosuppressive prophylaxis and the addition of corticosteroids. Acting via lympholysis, corticosteroids (alone or in combination with immunosuppressives) remain the most effective agent. Methylprednisolone at a dose usually of at least 2 mg/kg/day is initiated as primary therapy; overall grade of acute GvHD and graft source (HLA-sibling-match versus unrelated donor, etc.) may influence initial dosing decisions and subsequent response to therapy.

Patients who develop acute GvHD after allogeneic HCT and do not respond to primary therapy, usually with corticosteroids, often experience considerable morbidity and mortality [283, 297–299]. In a recent survey from the Swiss-Austrian-German GvHD consortium, the heterogeneity of second-line approaches used in different centers reflects this lack of standardization. The approaches to steroid-refractory (SR) acute GvHD range from increasing the dose of glucocorticoid therapy (if less than 2 mg/kg/day) to the addition of monoclonal antibodies such as alemtuzumab or polyclonal antibodies (antithymocyte globulin), including immunoconjugate toxins, additional immunosuppressive medications, and ECP [283, 297–300] (Table 57.10). Most such interventions that have shown success have been pilot studies that have not been corroborated in larger, randomized, controlled trials. For second-line treatment, randomized trials are missing. MMF has been studied in several trials but has not been demonstrated to significantly enhance overall patient outcomes. The addition of MMF to corticosteroids as initial therapy for acute GvHD does not improve GvHD-free survival compared with corticosteroids alone [301]. Failure during acute GvHD therapy often is due to the high incidence of opportunistic infections from bacterial, invasive fungal, and viral infections. As a result, all patients receiving systemic therapy for acute GvHD should receive intensive prophylaxis against infectious agents.

Chronic Graft-versus-Host Disease

Chronic GvHD, a significant cause of morbidity and mortality, occurs in about two-thirds of long-term survivors of allogeneic HCT [302–305]. As a result of greater availability

Table 57.10 Variety of second-line drugs/strategies utilized in acute G*v*HD treatment [300]

Drug	Mechanism of action	Toxicity
Mycophenolate Mofetil (CellCept®, Myfortic®)	Non-competitive inhibitor of IMPDH, the rate limiting step for de novo purine synthesis on which lymphocytes depend	Dose related cytopenia and gastrointestinal toxicity. Enteric-coated mycophenolic acid (Myfortic®) may be better tolerated.
Denileukin diftitox (Ontak®)	Recombinant fusion molecule of human IL-2 and diphtheria toxin that binds to the IL-2R-α and triggers apoptosis in activated T cells	Dose limiting toxicity -elevation of hepatic transaminases
Sirolimus (Rapamune®)	Binds to FK-binding protein complex and blocks mTOR, ultimately causing cell cycle arrest in G1.	Reversible cytopenia, hypertriglyceridemia, nephrotoxicity (HUS/TMA) and neurotoxicity (TTP). Less common are transaminitis, edema, arthralgias, and non-infectious pneumonitis
Infliximab (Remicade®)	Chimeric murine/human monoclonal antibody that binds with high affinity to soluble and membrane-bound TNFα, resulting in clearance of TNFα and T cells.	Anaphylaxis is uncommon, but can occur.
Etanercept (Enbrel®)	Soluble dimeric fusion protein that competes for TNF-α binding and renders it inactive	Generally well tolerated
Pentostatin (Nipent®)	Nucleoside analog that potently inhibits adenosine deaminase, reducing CD4 and CD8 T-cells and B-cells and lowering of IgG levels	Myelosuppression, should reduce dose by 50% if ANC <1000 per μL and discontinue if ANC < 500 μL. Reversible transaminitis, renal insufficiency and neurotoxicity can occur.
Daclizumab (Zenepax®)	Humanized monoclonal antibody against CD25 (IL-2 receptor) which prevents T cell proliferation	Well tolerated, anaphylaxis has not been observed.
Basiliximab	Chimeric (murine/human) monoclonal antibody against CD25 (IL-2 receptor) which prevents T cell proliferation	Anaphylaxis and hypersensitivity reactions have been reported but are not common.
Horse antithymocyte globulin (ATGAM®)	Antilymphocytic, primarily monomeric IgG, from hyperimmune serum of horses immunized with human thymus lymphocytes.	Anaphylaxis is uncommon but skin testing prior to first dose is recommended. Fever and chills common, also thrombocytopenia, leukopenia, and rash. Less common are serum sickness, dyspnea/apnea, arthralgia, chest, back, or flank pain; diarrhea and nausea and/or vomiting.
Rabbit antithymocyte globulin (thymoglobulin®)	Antilymphocytic globulin from hyperimmune serum of rabbits immunized with human thymus lymphocytes.	Skin testing is not considered necessary but must monitor closely for anaphylaxis or cytokine release syndrome. Thrombocytopenia and opportunistic infections are common.
Alemtuzumab (Campath®)	Humanized IgG1monoclonal antibody against CD52 on normal and malignant T- and B-cells, NK cells, monocytes, macrophages and some granulocytes.	Cytopenias including hemolytic anemia. Most common reactions are rigors and fever and nausea and vomiting. Less common are rash, fatigue, hypotension, urticaria, dyspnea, pruritus, headache, and diarrhea.
Ruxolitinib (Jakafi®)	Kinase inhibitor which selectively inhibits Janus Associated Kinases (JAKs), JAK mediated signaling involves recruitment of STATs (signal transducers and activators of transcription) to cytokine receptors which leads to modulation of gene expression	Cytopenia and cytomegalovirus-reactivation
Extracorporeal photopheresis (ECP)	Direct apoptosis of mainly lymphocytes and reinfusion interferes with dendritic cell maturation, cytokine modulation, and expansion of regulatory T cells	Limited, but includes blood loss from the extracorporeal circuit, hypocalcemia due to anticoagulant, and mild cytopenia.

Ho VT, Kekre N: Diagnosis and treatment of acute graft-versus-host disease. In: Abutalib SA, Hari. P (eds): Clinical Manual of Blood and Marrow Transplantation, Hoboken, NJ, Wiley-Blackwell, 2017 [300]

and use of alternative donors, increase in early posttransplant survival, inclusion of older recipients, and increasing use of nonmyeloablative conditioning regimens, the incidence of this syndrome appears to be rising [303]. Approximately 30–70% of subjects surviving >1 year after allogeneic HCT are affected and have a 5-year mortality rate of 30–50% [304, 305]. This syndrome is heterogeneous with respect to time of onset, affected organ sites, and response to treatment [306]. The variation in clinical presentation has made classification difficult. In contrast to acute GvHD that affects predominantly epithelial surfaces (skin, gastrointestinal tract, biliary radicals of the liver), chronic GvHD can affect both epithelial and mesenchymal tissues such as nerve and muscle as well as organs such as the buccal mucosa, lung,

and eye. The diagnosis of chronic GvHD often is challenging due to frequent coexistence of acute GvHD, polymorphic presentation, and lack of adequate biomarkers. Historically, chronic GvHD has been classified as subclinical (proven by histology but no clinical symptoms); limited extent (localized skin involvement, hepatic dysfunction due to chronic GvHD, or both); and extensive (generalized skin involvement, or limited extent plus involvement of a target organ such as liver histology showing chronic aggressive hepatitis, bridging necrosis, or cirrhosis, eye involvement, minor salivary gland, or oral mucosal involvement). Additionally, chronic GvHD has been classified according to the pattern of onset of clinical disease: de novo, quiescent, and progressive disease.

Newer classification systems have moved away from time of onset as a delineating feature of acute versus chronic GvHD and moved toward signs and symptoms at presentation [304, 305]. In addition, the conventional grading of chronic GvHD as limited versus extensive has been challenged. The newer NIH criteria for scoring of chronic GvHD provide meaningful information on disease severity and functional impairment of key organ systems in patients with chronic GvHD and are the current standard [304]. This scoring system can be applied *only after* the diagnosis of chronic GvHD has been established. While chronic GvHD is thought to result from immune dysregulation, the exact mechanisms remain unknown. There is evidence of the role of B-cell dysregulation, with production of autoreactive antibodies [307–310]. It has been suggested that B-cell activation may result from high levels of B-cell activating factor (BAFF) in the lymphoid microenvironment [307]. These observations may explain occasional responses of chronic GvHD to therapy with rituximab [311, 312].

The likelihood of developing chronic GvHD appears to be associated with increasing HLA disparity between recipient and donor (especially unrelated donor recipients), prior diagnosis of acute GvHD and corticosteroid use by 100 days after allograft, use of non-T-cell depleted bone marrow (compared to T-repleted bone marrow), male recipients of multiparous female donors, older age of recipient or donor, and use of blood rather than bone marrow grafts (Table 57.11) [86, 304, 313–316]. According to CIBMTR analysis the 1-year probability of chronic GvHD was approximately 30% after HLA-identical sibling HCT, 25–46% after one-antigen HLA-mismatched sibling HCT, and approximately 44–51% after HLA-matched unrelated HCT [317]. In addition, minor HLA antigen mismatches also may play a role in the development of chronic GvHD, particularly the H-Y antigen. Symptoms of chronic GvHD usually occur gradually, starting on average between 3 months and 2 years after HCT. Clinical manifestations of chronic GvHD are similar to autoimmune collagen vascular diseases including oral ulcerations (lichen planus),

Table 57.11 Risk factors for the development of chronic GvHD [322]

Most important risk factor	Prior acute GvHD
Frequently reported risk factors	Greater donor: recipient HLA disparity
	Increased donor and recipient age
	Use of female donors (especially multiparous) for male recipients
	Use of mobilized blood as a graft source
	Use of donor lymphocyte infusion
Less frequently reported risk factors	Higher infused $CD34^{+}$ cell dose in mobilized blood cell transplantation
	Lower infused $CD34^{+}$ cell dose when bone marrow is graft source
	Faster achievement of complete hematopoietic chimerism
	Corticosteroids in GvHD prophylaxis
	Transplantation for chronic myeloid leukemia and aplastic anemia
	Cytomegalovirus seropositivity in recipient

GvHD Graft-versus-Host Disease, *HLA* Human Leukocyte Antigen, *HPSC* hematopoietic progenitor and stem cells. Adapted from Pusic I and Pavletic SZ. Chronic Graft-versus-Host Disease- Updates, Challenges and Controversies. In: Lazarus HM et al. (eds) (2017) *Hematopoietic Cell Transplants: Concepts, Controversies and Future Directions*. Cambridge University Press, United Kingdom

keratoconjunctivitis sicca, xerostomia, polyserositis, esophagitis and stricture, vaginal ulceration and stricture, intrahepatic obstructive liver disease, obstructive pulmonary disease, scleroderma, morphea, fasciitis, and myositis [305]. Chronic GvHD diagnosis is primarily clinical and histologic confirmation is not mandatory if the patient has at least one diagnostic sign. Further testing is recommended to confirm chronic GvHD in situations where distinctive but non diagnostic signs are present or alternative diagnoses are considered. A detailed color-atlas and classification of the skin and oral chronic GvHD is available at http://www.asbmt.org/?page=MeasureCGvHD [305]. In the absence of histologic or clinical signs characteristic of chronic GvHD, persistence, recurrence or new onset of maculopapular erythematous rash, gastrointestinal symptoms or cholestatic hepatitis should be classified as acute GvHD, regardless of the time after transplantation. The 2014 NIH consensus criteria have defined the *overlap* category as the presence of acute GvHD manifestations in a patient diagnosed as being affected with chronic GvHD. The *overlap* syndrome is often transient and depends on degree of immunosuppression. In those patients who present with the *overlap* syndrome, acute GvHD features can resolve with treatment while chronic GvHD symptoms and signs may persist. Likewise, acute GvHD may flare in patient with chronic features when immunosuppression is tapered.

Currently, there are no preventive strategies shown to improve overall survival in patients with chronic GvHD. Only antithymocyte globulin appears to decrease incidence of

chronic GvHD and chronic lung dysfunction without providing a survival benefit [316, 318, 319]. While decreasing the incidence of acute GvHD, T-cell depletion of the graft has not been shown to lower the incidence of chronic GvHD as demonstrated in a randomized, multicenter trial [319].

Effective treatment for chronic GvHD has evolved little over the past several years [320–322]. Immunosuppressive therapy, initially with corticosteroids, with the addition of cyclosporine or tacrolimus, is a generally accepted regimen for those patients needing therapy. A study examining the addition of MMF failed to demonstrate any benefit [323]. About 50% of patients with chronic GvHD will fail to achieve complete or partial response to steroid therapy by 1 year after diagnosis. As there is no standard of care for second-line therapy for chronic GvHD, when possible, patients should be treated on clinical trials. Second-line therapy is indicated for patients who fail to respond or progress through steroid-based therapy (Table 57.12) [321, 322]. A number of small phase II studies have been published, most of them showing incomplete and brief responses. Although the mechanism of action is unknown, there are recent, promising results using rituximab [311, 312], ECP [281], imatinib [324, 325], and ruxolitinib [326] for treatment of sclerotic chronic GvHD. Preliminary results presented in abstract form of a phase II trial with ibrutinib (n = 42) demonstrated multiorgan responses in two-thirds of the patients [Blood. 2016;128(22)]. Based on these data, Ibrutinib was approved by the US Food and Drug Administration (FDA) for treatment of chronic GvHD and is an acceptable alternative to calcineurin inhibitors.

Organ dysfunction and opportunistic infection may accompany chronic GvHD [327]. Hence, ancillary therapy

Table 57.12 Second-line therapies for chronic GvHD [322]

Agent	Mechanism of action	Common and serious side effects	Comments
Pentostatin	Nucleoside analog	Nausea, vomiting, infections, renal	Activity in treatment of both acute and chronic GvHD
Low-dose methotrexate	Antimetabolite, anti-inflammatory	Myelosuppression, liver toxicity	Cannot be used in renal failure
Imatinib	Multi-kinase inhibitor targeting PDGFR and TGF-β	Myelosuppression, hepatitis	Best responses in skin and pulmonary GvHD
Psoralen and ultraviolet A	8-Methoxypsolaren and ultraviolet A irradiation	Nausea, photosensitivity, skin cancers	No systemic effect, best for skin lichen-planus lesions
Hydroxychloroquine	Anti-malarial agent	Gastrointestinal, renal polyneuropathy	Best results in mucocutaneous and liver GvHD
Montelukast	Leukotriene antagonist	Headache, nausea	Lung GvHD
Acitretin	Retinoid	Skin dryness, alopecia, night blindness, pancreatitis, hyperlipidemia	Skin GvHD
Pulse cyclophosphamide	Alkylating agent	Myelosuppression, hemorrhagic cystitis	Effective for skin, liver, oral GvHD; toxicity profile acceptable
Clofazimine	Antimycobacterial	GI symptoms, hyperpigmentation	Best responses in mucocutaneous GvHD
Total lymphoid irradiation	Low-dose 100 cGy ionizing irradiation	Leukopenia, infections	Best responses in fasciitis and oral GvHD; rarely used today
Bortezomib	Proteasome inhibitor	Diarrhea, neuropathy	Small studies, responses in GI and skin GvHD
Daclizumab	Humanized anti-IL-2 receptor monoclonal antibody	GI symptoms, headache, insomnia, fatigue, infections	Limited reports, responses in skin GvHD
Infliximab	Chimeric anti-TNF-a monoclonal antibody	Hypersensitivity, infections	Limited reports
Etanercept	Recombinant human soluble TNF receptor fusion protein	Infections	Small studies, use in overlap syndrome with GI and skin GvHD
Low-dose IL2	Cytokine; induces Treg expansion	Constitutional symptoms, thrombotic microangiopathy	Best responses in skin and joint GvHD
Thalidomide	Anti-inflammatory, immunomodulatory	Neuropathy, somnolence, constipation, neutropenia, teratogenic, DVT	Disappointing as primary therapy; toxicity is a limitation
Alefacept	Dimeric anti-CD2 LFA-3 fusion protein	Infectious risk	Limited reports, last resort
Alemtuzumab	Unconjugated IgG1 monoclonal antibody	Infectious risk	Limited reports, last resort

GvHD Graft-versus-Host Disease, *GI* gastrointestinal, *DVT* deep venous thrombosis, *Treg* regulatory T-cell. Adapted from: Pusic I, Pavletic SZ. Chronic Graft-versus-Host Disease- Updates, Challenges and Controversies. In (eds) Lazarus HM et al. (2017) *Hematopoietic Cell Transplants: Concepts, Controversies and Future Directions*. Cambridge University Press, United Kingdom [322]

Table 57.13 Ancillary therapy and supportive care interventions in chronic GvHD [327]

Organ system	Organ-specific intervention
Skin and appendages	Photoprotection, sunscreens, avoidance of sun exposure; surveillance for malignancy
	For intact skin: topical emollients (ointments and creams are better than lotions), corticosteroids (Triamcinolone cream 0.1% tid to body; Hydrocortisone cream 1.0% bid to face, axila and groin), antipruritic agents, PUVA, calcineurin inhibitors
	For erosions/ulcerations: topical antimicrobials, protective films or other dressings, debridement, hyperbaric oxygen, wound care specialist consultation
Mouth and oral cavity	Maintain good oral/dental hygiene. Routine dental cleaning and endocarditis prophylaxis. Surveillance for infection and malignancy. Nutritional counseling.
	Topical high and ultra-high potency corticosteroids (fluocinonide gel 0.05%, triamcinolone 0.1%), analgesics (mouthwash- 1 part dexamethasone +2 parts viscous lidocaine, follow by tap water rinse), topical tacrolimus ointment 0.1%. Therapy for xerostomia (fluoride, saliva substitutes, cevimeline, pilocarpine)
Eyes	Photoprotection, regular ophthalmology exams by eye specialist
	Artificial tears and ointments, topical corticosteroids or cyclosporine, punctal occlusion, humidified environment, occlusive eye wear, moisture chamber glasses, tarsorrhaphy, gas-permeable scleral contact lens, autologous serum, topical antimicrobials, doxycycline.
Musculo-skeletal	Surveillance for decreased range of motion, yearly bone densitometry, calcium levels and 25-OH Vitamin D
	Physical therapy, calcium, vitamin D, bisphosphonates
Gastrointestinal tract and liver	Surveillance for infection (viral, fungal)
	Eliminate other etiologies. Dietary modification, enzyme supplementation for malabsorption, gastroesophageal reflux management, esophageal dilatation, pancreatic enzyme replacement for pancreatic insufficiency, topical corticosteroids, bile salts resins
Lungs	Surveillance for infection
	Eliminate other etiologies. Inhaled glucocorticoids, bronchodilators, supplementary oxygen. Pulmonary rehabilitation. Consider lung transplantation for appropriate candidates
Hematopoietic	Eliminate other etiologies, surveillance for infection
	Hematopoietic growth factors, immunoglobulin
Neurologic	Calcineurin drug level monitoring. Seizure prophylaxis including blood pressure control, electrolyte replacement, anticonvulsants
	Occupational and physical therapy, treatment of neuropathic syndromes with tricyclic antidepressants, selective serotonin reuptake inhibitors, or anticonvulsants
Vulva and vagina	Surveillance for estrogen deficiency, infection and malignancy
	Water-based lubricants, topical estrogens, topical corticosteroids or calcineurin inhibitors, dilators, surgery for extensive synechiae or obliteration
Immunologic and infectious disease	Immunizations and prophylaxis against PCP, VZV and encapsulated bacteria. Consider immunoglobulin replacement. Surveillance for infection.
	Organism-specific antimicrobials and empiric broad-spectrum antibiotics for fever

Modified from Carpenter PA, et al. NIH Consensus Development Project on Criteria for Clinical Trials in Chronic Graft-versus-Host Disease: V. The 2014 Ancillary Therapy and Supportive Care Working Group Report. Biol Blood Marrow Transplant. 2015; 21: 1167–1187 [327]

and supportive care are the central components in the long-term multidisciplinary management of affected patients (Table 57.13). Immunodeficiency, leading to a variety of opportunistic infections and often to death, is the most important associated complication of chronic GvHD [327]. *Pneumocystis cariini*, *Streptococcus pneumoniae*, and CMV are the pathogens most likely to cause serious and fatal infection, and prophylaxis strategies against these pathogens should be undertaken. Comprehensive guidelines for infectious prophylaxis in HCT recipients, including patients with chronic GvHD, have been published [327]. Patients experiencing chronic GvHD, with or without attendant immunosuppression, likely may not have an optimal response to anti-infective vaccination (see below).

Late Complications

As a result of many biologic and technologic advances, tens of thousands of HCT recipients are alive today [31, 328–333]. The HCT procedure is associated with considerable early morbidity and mortality but those who attain long-term survival generally enjoy good health. Many of these individuals no longer are under the care of the physicians at the transplant center and are followed by health care providers in the community who are not necessarily familiar with late complications. Such conditions after transplant include those due to the preparative regimen, late infections, chronic GvHD, recurrence of malignancy, and secondary malignancy. Such complications include dental defects/caries, cataracts and other ophthalmic complications, osteoporosis/osteopenia,

avascular bone necrosis, late neurologic conditions such as leukoencephalopathy and cognitive function impairment, retardation of growth and development in children, endocrine disturbances such as hypothyroidism, infertility, hypogonadism, restrictive lung disease, chronic obstructive pulmonary disease, bronchiolitis obliterans organizing pneumonia (cryptogenic organizing pneumonia), iron overload, hepatitis B and C infection, and psychologic and sexual dysfunction [31, 333, 334]. Infertility, which can be devastating to young patients, is almost universal when using myeloablative regimens. This can be addressed with sperm cryopreservation for male patients, assuming they have adequate sperm number and function. Embryo or oocyte cryopreservation in female patients can also be attempted, with generally less favorable results [331].

In an effort to provide a uniform and thorough approach, a consensus panel formulated screening and preventative practices [31, 332, 333]. Major areas include addressing visceral organ (pulmonary, cardiovascular, renal) complications, secondary malignancy, and anti-infective vaccinations. Some investigators have subcategorized late complications into (a) delayed (onset at 3 months to 2 years and often affecting lung due to chronic GvHD); (b) late (onset at 2–10 years, often calcineurin-induced renal injury); and (c) very late events (onset at >10 years, frequently cardiac dysfunction due to anthracycline exposure) [31, 334]. In most instances, these recommendations are not based on randomized or controlled clinical trials. Nonetheless, these approaches represent a sensible practice for optimizing long-term outcome. Patients should be evaluated at least once or twice a year for conditions common in the general population including essential hypertension, hypercholesterolemia, diabetes mellitus, sexually transmitted diseases and depression, as well as other conditions [31]. When indicated, sophisticated diagnostic testing should be undertaken, including invasive studies/biopsies, if necessary.

Quality of Life After Hematopoietic Cell Transplant

Over the past three decades, the primary focus of autologous and allogeneic HCT has been to improve overall survival by eliminating the underlying disorder and reduce the immediate and early complications. Greater success of this modality has produced many more survivors and correspondingly, health care personnel have placed an increasing emphasis on the patient's quality of life (QoL) after the transplant. In fact, cancer survivors routinely cite QoL as among their greatest concerns [335, 336]. QoL is a dynamic, multifaceted concept related to physical, cognitive, emotional, and social and financial functioning and well-being [335, 336]. Several factors, including the continued risk of relapse, however, negatively may affect a transplant survivor's ability to function fully and normally in important areas of everyday life, including employment, relationships with others, and sexuality.

A number of publications have addressed QoL evaluation after transplant [337–340]. These and other studies show the most significant issues interfering with QoL are altered body image, sexual difficulties, fatigue, lack of physical strength, inability to work at their chosen employment, and financial problems. The psychosocial literature that examines patients longitudinally indicates faster return to baseline for autograft compared to allograft recipients. Subjects with moderate impairment after transplant generally return to baseline function by 100 days after HCT and that more than half of patients report good to excellent QoL with no major functional limitation when queried 1–2 years after transplant. On the other hand, approximately one-quarter of patients still have ongoing, significant, or bothersome symptomatology. Many of the physical and psychosocial deficits perceived as problems by the patient were, in fact, present prior to transplant. A lower QoL persists more often in older patients, those with less formal education, subjects with a more advanced disease state at transplant, and those in whom there is evidence of active chronic GvHD or chronic visceral organ dysfunction. Progress in this area is hampered by lack of standardized instruments that would facilitate comparisons within HCT groups and with non-HCT groups. Further, transplant physicians need to better understand patients' symptoms and QoL issues in order to facilitate progress [341, 342].

Second Malignancies After Hematopoietic Cell Transplants

All patients undergoing HCT should be informed of the potential risk of developing a second cancer and should be monitored on a continuous basis [31, 332]. The trend toward an increase in risk over time after transplantation and the greater risk among younger patients, particularly those who received autologous transplant, indicates the need for lifelong surveillance. HCT survivors, however, had similar health behaviors as matched controls and comparable to those reported by cancer survivors who did not undergo HCT despite higher levels of engagement with health care providers [334]. Several groups have reported their experiences to determine the incidence and risk factors for the development of new malignancies occurring after HCT. Baker and coworkers [343] noted 147 posttransplant malignancies in 137 patients (including myelodysplasia, acute leukemia, lymphoproliferative disorders, and solid tumors) from a population of 3372 who underwent HCT during the period 1974–2001. This figure represented an eightfold increased

risk and a 6.9% cumulative incidence for the development of any post-HCT malignancy at 20 years. The cumulative incidence plateaued at 1.4% by 10 years for both posttransplant lymphoproliferative disorders, myelodysplasia and acute myeloid leukemia. Solid tumor incidence, on the other, continued to increase (3.8% at 20 years), necessitating long-term, close follow-up.

In one of the largest studies, Rizzo and colleagues from the CIBMTR retrospectively studied 28,874 allogeneic transplant recipients and noted 189 solid tumor malignancies, twice the expected rate [344]. The risk for non-squamous cell cancers was increased substantially in patients younger than age 30 years who received radiation as part of conditioning while chronic GvHD and male gender were the main determinants for risk of squamous cell carcinomas. These data underscore the need to develop strategies to promote lifelong surveillance among these patients.

Risk factors for secondary malignancy include radiation therapy, length/severity of immunosuppression, and chronic GvHD [31, 345, 346]. Recommendations for screening include skin and breast self-examination, avoidance of tobacco and UV exposure. Further, general health maintenance testing should be performed as in the general population, including screening for essential hypertension, hypercholesterolemia, colorectal cancer, diabetes mellitus, depression, sexually transmitted diseases, prostate cancer in men, and breast and cervical cancer in women [31].

Infection Prevention and Posttransplant Vaccination

Patients undergoing both autologous as well as allogeneic HCT are exposed to cytotoxic and immunosuppressive agents. Further, the hematopoietic grafts are in the process of regeneration and often the patients have GvHD or are receiving treatment for this syndrome. These factors, alone or in combination, contribute to a heightened and prolonged risk for the development of many opportunistic infections by bacteria, fungi, viruses, and even protozoa [347, 348]. In addition, HCT often is associated with a loss or diminution of previously acquired host protective immunity against pathogens targeted by childhood immunizations such as tetanus, mumps, rubella, and poliovirus [349, 350]. Vaccine efficacy in HCT recipients has not generally been studied in large multicenter trials and previously published surveys in Europe and the United States varied considerably in the posttransplant vaccination practice guidelines. The current guidelines now are more uniform. Vaccination recommendations include consideration of initiating anti-infective vaccinations as early as 6 months after HCT, predominantly in patients no longer receiving immunosuppressants. Anti-infective vaccines include use of protein-conjugated pneumococcal vaccine (in contrast to the less immunogenic polysaccharide vaccine), tetanus, diphtheria, acellular pertussis vaccine, *Hemophilus influenza* type b conjugate, meningococcal conjugate, inactivated poliovirus, inactivated influenza, and recombinant hepatitis B vaccine. Human papillomavirus (HPV) is causally linked to squamous cell carcinoma of the genital tract and head and neck; the cancer incidence is higher among HPV infected and immunosuppressed individuals. Several groups recognize the value of vaccination against HPV and recommend use in HCT recipients [351, 352].

Most anti-infective vaccine preparations are devoid of live organisms, the previous exception being a live, attenuated mumps-measles-rubella vaccine. More recently, the recommendations have been expanded to permit use of a dose-attenuated, live, varicella-zoster virus vaccine (Varivax®) in patients beyond 2 years after HCT who are not receiving immunosuppressives [353]. The robust-dose varicella-zoster virus vaccine Zostavax®, however, is contraindicated. Other vaccinations not in routine use, to be given only under special circumstances, include hepatitis A, yellow fever (live, for use in endemic areas), rabies (occupational exposure), tick-borne encephalitis, and Japanese B encephalitis, both for endemic areas. Those vaccines that should be avoided include Bacillus Calmette-Guérin (live), oral poliovirus (live), intranasal influenza (live), cholera, typhoid (both oral, live and intramuscular), and rotavirus. Testing after vaccination is indicated to determine response and the need for additional or boost vaccination. For the vaccine to be relevant clinically, a protective immune response in the form of at least a fourfold rise in specific antibody titers, or T- and B-cell adaptive immunity must be partially reconstituted. In some instances, vaccination using tetanus toxoid, pneumococcal conjugate vaccine, and *H. influenza* type-b conjugate vaccine of the potential hematopoietic cell donor has been shown to improve the posttransplant immunity of the graft recipient. The logistics for this approach, however, are quite complex and no specific recommendations can be made.

Summary

HCT has become a frequently used tool to provide potentially curative therapy for a variety of neoplastic, immunologic, and genetic disorders in both adults and children. As a result of previous and ongoing clinical and translational work, many of the hurdles that formerly plagued this approach have been lowered or removed and treatments can be individualized for each potential recipient. Vigorous supportive care with blood component transfusions, broad-spectrum antibiotics, and immunosuppressives to prevent or treat GvHD remain an essential component of this life-saving art. While many thousands of patients have achieved cure or

significant prolongation of survival, work still remains and participation in clinical trials is strongly encouraged to continue the dramatic progress.

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58 Hematopoietic Growth Factors in the Supportive Care and Treatment of Patients with Hematologic Neoplasms

Ryan W. Jacobs, Omotayo Fasan, Edward A. Copelan, and Belinda R. Avalos

Introduction

The hematopoietic growth factors (HGFs) are naturally occurring glycoproteins required for the survival, proliferation, and differentiation of hematopoietic cells. Their effects are mediated by high-affinity binding to specific receptors on their hematopoietic target cells. The isolation, purification, cloning, and manufacture of HGFs have permitted their clinical use, originally to correct cytopenias caused by deficiency of specific growth factors, and subsequently in additional settings. Recombinant HGFs are used in the treatment of patients with hematologic malignancies to improve disease and treatment-related cytopenias and to mobilize hematopoietic stem cells from the marrow into the peripheral blood from where they can be harvested for autologous and allogeneic hematopoietic cell transplantation. This chapter will focus on the biology and use of the clinically important recombinant HGFs in patients with hematologic malignancies. Those HGFs which are employed clinically will be individually discussed, including a description of their application in specific clinical settings.

Clinically Important Hematopoietic Growth Factors

Granulocyte Colony-Stimulating Factor

Granulocyte colony-stimulating factor (G-CSF), also called CSF3, is a naturally occurring glycoprotein. The gene that encodes it is located at chromosome 17q21-22 and the transcribed molecule exists in two forms, 174 and 177 amino acids long. G-CSF is responsible for the regulation of granulopoiesis promoting the maturation of myeloid progenitor cells along the polymorphonuclear neutrophil (PMN) lineage [1].

G-CSF supports the survival, proliferation, and differentiation of neutrophil precursors and the survival and activity of mature neutrophils. In particular, it upregulates the expression of C3b receptors, increases the binding of the chemotactic agent N-formyl-Met-Leu-Phe (fMLP) to PMNs, enhances generation of superoxide anions, and promotes antibody-dependent cellular cytotoxicity (ADCC) and the release of arachidonic acid (a source of proinflammatory and anti-inflammatory eicosanoids) [2–6]. G-CSF null mice demonstrate chronic neutropenia and severely impaired responses to infection [6].

The biological activities of G-CSF are mediated by the GCSF receptor (CSF3R), a transmembrane protein expressed on the surface of cells of the neutrophil lineage [7, 8]. Like other cytokine receptors, the extracellular portion of CSF3R binds ligand and the cytoplasmic tail transduces intracellular signals [3, 4, 7]. Distinct regions in the cytoplasmic domain mediate specific activities. The membrane-proximal domain of CSF3R mediates proliferative signals, while the distal region transduces differentiation signals. Studies of mice with CSF3R knock-out or knock-in mutations indicate the CSF3R generates unique signals to maintain circulating PMN levels during basal and stress granulopoiesis [9–12]. CSF3R null mice demonstrate chronic neutropenia, decreased numbers of myeloid cells in the bone marrow, and defects in PMN activation [6, 10]. A total of five different CSF3R isoforms differing in their transmembrane or cytoplasmic sequences and arising from alternative splicing have been isolated in humans. Most notable is the Type IV isoform that replaces the 87 carboxy-terminal amino acids of the full-length Type I CSF3R with a unique sequence and is defective in differentiation signaling [13–17].

In 1991, recombinant human G-CSF became the first CSF to receive FDA approval in the United States. G-CSF (filgrastim, Neupogen) was initially approved for prevention of febrile neutropenia in patients with cancer receiving myelosuppressive

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chemotherapy after a Phase III placebo-controlled trial demonstrated a reduction in the incidence of febrile neutropenia (57% vs. 28% in cycle 1), the duration of neutropenia, days of hospitalization, and days of intravenous antibiotic use [18]. Additional studies have consistently shown a significantly shorter duration of neutropenia with G-CSF than in controls. The labeled indications for G-CSF were subsequently expanded to include use for accelerating neutrophil recovery after bone marrow transplantation, mobilizing progenitor cells into the peripheral blood for collection and hematopoietic cell transplantation, and shortening the duration of neutropenia in patients receiving induction and consolidation chemotherapy for acute)myelogenous leukemia (AML). G-CSF is also approved to reduce the incidence and sequelae of neutropenia in symptomatic patients with congenital, cyclic, or idiopathic neutropenia and in treating patients acutely exposed to myelosuppressive doses of radiation.

Concerns regarding the appropriate use of high-cost CSFs prompted the American Society of Clinical Oncology (ASCO) to convene a panel of experts to review data evaluating the effectiveness of CSFs and develop practice guidelines for the use of CSFs in patients not enrolled in clinical trials, which were published in 1994 [19] and updated in 1996 [20], 2000 [21], 2006 [22], and 2015 [23]. The 2015 guidelines support primary prophylaxis with G-CSF starting with the first cycle and continuing with subsequent cycles of use of myelotoxic chemotherapy when the risk of febrile neutropenia is estimated to be 20% or higher. The recommended dose in this setting is 5 μg/kg/day subcutaneously beginning 1–3 days after administration of chemotherapy. If G-CSF is administered after high dose chemotherapy and autologous stem cell rescue it can be started 1–5 days after chemotherapy. G-CSF should generally be continued until the ANC is greater than $2–3 \times 10^9$/L. In patients who do not receive primary prophylaxis, secondary prophylaxis with G-CSF should be considered if they experience neutropenic fever in the preceding cycle, especially if delayed treatment or dose reduction may adversely affect survival. The routine administration of G-CSF to patients with neutropenia following chemotherapy should be avoided in the absence of fever. Febrile neutropenic patients in high-risk situations such as those with severe neutropenia, i.e., ANC < 0.1 × 109/L with expected duration of neutropenia of 10 days or more, elderly (age > 65 years) or critically ill patients with pneumonia, invasive fungal infection or multiorgan failure and sepsis syndrome should receive G-CSF at a dose of 5 μg/kg daily.

G-CSF is utilized for peripheral blood progenitor cell mobilization either alone, in combination with plerixafor, a CXCR4 antagonist, or after chemotherapy. The usual dose is 10 μg/kg daily for at least 4 days before apheresis and continuing daily until the target progenitor cell dose is reached. Some mobilization algorithms administer an extra dose of filgrastim on the evening of day 4 while some employ twice daily dosing at 8 μg/kg twice daily.

The utilization of G-CSF in patients with acute myelogenous leukemia has been controversial, because G-CSF stimulates the proliferation of leukemic blasts in vitro. However, G-CSF has not demonstrated an adverse effect on complete remission duration or on survival of AML patients who receive it compared to those that do not where the leukemic burden has been reduced by cytotoxic chemotherapy. In fact G-CSF significantly accelerates the recovery of neutrophils after chemotherapy variably resulting in a decrease in the incidence of infections, the number of days of febrile neutropenia, duration of infection, and antibiotic use. Some studies, however, failed to show a decreased incidence of infection. There is some evidence that the resurgence of leukemic blast cells may occur in patients whose blast cells have fewer numbers of G-CSF receptors with a high affinity [24–26].

In acute lymphocytic leukemia (ALL), use of CSFs is recommended after the first few days of initial induction therapy or following the first post-remission course. The use of G-CSF in)children with ALL, however, should be carefully considered due to their excellent prognosis and the potential risk of secondary myeloid leukemia and myelodysplasia (MDS).

Pegylated G-CSF (pegfilgrastim, Neulasta) [it is a long-acting FDA-approved G-CSF] is a modified filgrastim in which a 20 kDa polyethylene glycol (peg) moiety is covalently added to the N-terminus of G-CSF. The addition of the peg moiety decreases clearance, particularly by the kidneys, resulting in sustained drug levels. Pegfilgrastim and filgrastim have an identical mechanism of action, but because pegfilgrastim has a longer half-life, it can be dosed once per chemotherapy cycle to prevent neutropenia. The recommended dose of pegfilgrastim in adults is 6 mg once to be given more than 24 h after the completion of chemotherapy. Pegfilgrastim has also been utilized for peripheral blood progenitor cell mobilization. In a cohort of 52 patients with hematologic malignancies single dose pegfilgrastim 6 mg ($n = 2$) or 12 mg ($n = 20$) was compared to filgrastim (G-CSF) 10 μg/kg daily from day 1 with apheresis beginning when the peripheral blood CD34 (PBCD34) reached a threshold of >5/μL on day 4 or day 5. The median PBCD34 count was 26/μL in the pegfilgrastim group compared to 16.2/μL in the filgrastim group. The target CD34 yield ($>2 \times 10^6$ per kg) was reached in 91% vs 80% in the pegfilgrastim and filgrastim arms, respectively, within a similar median of 3 days of apheresis in both arms [27].

Following the expiration of Amgen's patent on filgrastim (Neupogen), numerous G-CSF biosimilars have become available for clinical use worldwide. The approval of biosimilars occurs by an abbreviated process for biologics shown to be interchangeable or "biosimilar" to a reference standard. In March 2015 the FDA approved Sandoz's Zarxio (filgrastim-sndz) a biosimilar to Amgen's Neupogen, the reference standard. Zarxio is approved for similar indications to Neupogen (patients with cancer receiving myelosuppressive chemotherapy, patients with acute myeloid leukemia receiving induction or consolidation chemotherapy,

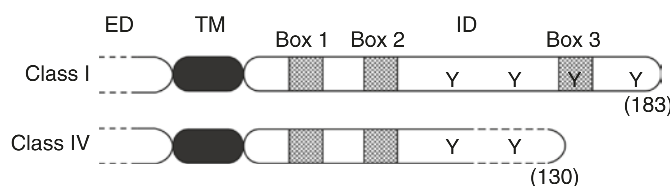

Fig. 58.1 Schematic diagram of Class I (wild-type) and Class IV CSF3R isoforms. The extracellular domain (ED), transmembrane domain (TM), and intracellular domain (ID) are indicated. The length of the intracellular domain is indicated by the number in parentheses at the bottom right. The locations of the cytoplasmic tyrosine (Y) residues at positions 704, 729, 744, and 764 are indicated. The dotted line represents the alternative carboxyl tail in the Class IV G-CSFR within which lies a unique tyrosine residue at position 734 (modified with permission from Avalos BR. Molecular analysis of the granulocyte colony-stimulating factor receptor. Blood 1996;88:761–77)

patients undergoing bone marrow transplantation, patients undergoing peripheral blood progenitor cell mobilization and collection, and patients with severe chronic neutropenia).

Common adverse events due to filgrastim and pegfilgrastim include nausea, headaches, mild to severe bone pain, and myalgias. Notable severe adverse events include anaphylaxis, splenic rupture, sickle cell crisis (when administered to patients with sickle cell disease), capillary leak syndrome, and acute respiratory distress syndrome. More recently the FDA highlighted the occurrence of a reversible glomerulonephritis with hematuria, proteinuria, and azotemia. A dose reduction or cessation of G-CSF should be considered if this develops.

Acquired point mutations in the CSF3R gene resulting in truncation of the carboxy-terminal maturation-inducing region of the CSF3R have been reported in patients with AML with preceding severe congenital neutropenia (SCN), an inborn disorder of granulopoiesis frequently associated with mutations in the gene encoding neutrophil elastase [28]. SCN has a marked propensity for transformation to MDS/AML with a cumulative transformation rate that exceeds 20%. Despite the relatively high frequency (30%) of mutations in tyrosine kinase genes such as FLT3, JAK2, and KIT in patients with de novo AML, these mutations have not been detected in patients with SCN transforming to AML [29]. Instead, mutations of the CSF3R gene are common and reported in up to 78% of patients with SCN and monosomy 7, MDS, or AML in one series [30]. There is evidence that the truncation mutations of CSF3R observed in patients with SCN are activating. Expression of the mutant CSF3R forms in myeloid cell lines results in defective ligand internalization and enhanced proliferative and survival signals in response to G-CSF [31, 32]. Transgenic mice carrying truncating mutations of CSF3R also display a hyperproliferative response to G-CSF [9, 11]. Since the CSF3R and tyrosine kinases share many signaling pathways, these observations along with the rarity of tyrosine kinase mutations in SCN/AML suggest the mutant CSF3R may provide the missing activated tyrosine kinase signal for malignant transformation. Notably, in patients with SCN less responsive to even high doses of G-CSF (≥8 μg/kg/day) there is a significantly increased risk of transformation to AML/MDS compared to SCN patients responsive to lower doses of G-CSF [33] (40% vs. 11% after 10 years).

The use of G-CSF with immunosuppressive therapy in severe aplastic anemia (SAA) remains under study and is currently not standard treatment. Previous studies by several groups have suggested a link between the use of G-CSF and an increased risk of MDS/AML after immunosuppressive therapy in patients with SAA. In the largest survey published, the European Group for Blood and Marrow Transplantation (EBMT) reported that the incidences of MDS/AML in 840 patients with SAA receiving immunosuppressive therapy who did or did not receive G-CSF were 10.9% and 5.8%, respectively. A significantly higher hazard (1.9) of MDS/AML was associated with the use of G-CSF, and the use of G-CSF did not improve overall survival [34]. Whether G-CSF provides a survival advantage to an abnormal hematopoietic stem/progenitor cell clone in the subset of patients developing MDS/AML remains unknown (Fig. 58.1).

Granulocyte–Macrophage Colony-Stimulating Factor

Granulocyte–macrophage colony-stimulating factor (GM-CSF) is a pleiotropic cytokine secreted by T cells, macrophages, mast cells, endothelial cells, and fibroblasts in response to inflammatory stimuli and cytokines. Recombinant human GM-CSF supports the growth of progenitors of multiple lineages including granulocytes, monocytes, eosinophils, and megakaryocytes [35–37]. The predominant effect of GM-CSF is increased production of neutrophils and macrophages, but GM-CSF is also a potent chemoattractant for neutrophils and eosinophils and enhances the effector functions of neutrophils and macrophages [38]. More recently, GM-CSF has been shown to function as a dendritic cell growth factor and to stimulate effector functions of dendritic cells [39, 40]. The broad spectrum of GM-CSF's activities likely explains its toxicity profile, which includes fever, chills, hypersensitivity reactions, and manifestations of capillary leak syndrome with generalized edema, weight gain, and pleural and pericardial effusions.

GM-CSF exerts its biological activities by binding to specific receptors on the surface of responsive cells. Unlike the receptor for G-CSF which is a single chain that dimerizes upon ligand binding, the GM-CSF receptor is more complex

and composed of a ligand-specific α chain subunit and a common signal-transducing beta subunit that is shared with the receptors for interleukins-3 and -5 (IL-3 and IL-5). The complexity of the GM-CSF receptor contributes to its diverse biological activities [41].

Recombinant human GM-CSF (sargramostim) is a glycoprotein made from a Saccharomyces cerevisiae expression system. It is 127 amino acids long and the sequence differs from the naturally occurring molecule at position 23 where an arginine molecule takes the place of leucine. It initially received FDA approval in March 1991 for use following autologous bone marrow transplantation, following a placebo-controlled multicenter trial of high dose chemotherapy and autologous transplantation for lymphoid malignancies in which patients randomized to receive GM-CSF 250 μg/m^2/day experienced neutrophil recovery 7 days earlier than those not receiving G-CSF (19 days vs. 26 days) [42]. GM-CSF use was associated with fewer days of hospitalization and antibiotic administration, and a lower incidence of infection. GM-CSF was subsequently approved for use in patients experiencing graft failure or delayed engraftment in both the autologous and allogeneic settings as well as for AML, mobilization of peripheral blood stem cells, and following peripheral blood progenitor cell transplantation.

Erythropoietin

Erythropoietin is a glycoprotein that promotes the proliferation of erythroid progenitors and supports the survival of erythroid cells. The existence of erythropoietin was first suspected when investigators injected serum from anemic rabbits into normal animals and noted an increase in the number of reticulocytes which peaked at about 5 days [43]. It is produced predominantly by the juxtatubular interstitial cells of the renal cortex where an oxygen-dependent hydrolase regulates the stability of hypoxia-inducible factor 1α (HIF-1α), the primary transcription factor for erythropoietin [44]. Low oxygen levels maintain inactive hydrolase and stable, unhydroxylated HIF-1α, which drives production of erythropoietin [44, 45]. Blood levels, which are normally approximately 20 mU/mL, increase with declining tissue oxygenation accompanying anemia or hypoxia to levels as high as 200,000 mU/mL [46].

Exogenously administered erythropoietin increases the number of erythroid progenitor cells in the marrow, where it promotes red blood cell (RBC) production and exit into the bloodstream. It was initially approved for treatment of anemia due to chronic renal failure, which is associated with erythropoietin deficiency. It has been subsequently applied in anemias of multiple etiologies. Recombinant preparations are as potent as naturally occurring erythropoietin. The addition of an N-linked carbohydrate (darbepoetin) prolongs its half-life [47]. The American Society of Clinical Oncology/American Society of Hematology clinical practice guideline update consider the effectiveness and safety of these agents to be equivalent [48].

Anemia is common in patients with hematologic malignancies as a consequence of disease and therapy-related suppression of hematopoiesis. Anemia of chronic inflammation, blood loss related to thrombocytopenia and/or frequent blood draws, immune mechanisms, and other factors may contribute as well. Patients with anemia and malignancy often do not produce amounts of erythropoietin appropriate for their degree of anemia [49]. Those whose blood level exceeds 500 mU/mL are less likely to benefit from exogenous administration than patients whose levels are low [50], but, aside from myelodysplastic syndromes (MDSs), levels are not considered sufficiently predictive to guide treatment. Controlled trials demonstrate that patients with cancer and hemoglobin <10 g/dL require fewer blood transfusions and feel better with erythropoietin therapy [47, 48].

ASCO and ASH initially published evidence-based practice guidelines for the use of erythropoietin in 2002 [51] and subsequently updated them [48, 52]. These guidelines emphasize consideration of other correctable causes of anemia before initiating therapy with erythropoiesis-stimulating agents (ESAs). Appropriate clinical evaluation for correctable causes include a history, physical examination, and diagnostic tests [52]. The use of epoetin or darbepoetin is recommended as a treatment option in individuals with anemia associated with chemotherapy whose hemoglobin concentration is less than 10 g/dL in order to reduce the transfusion of red blood cells. Clinical judgment should determine when to implement treatment in individuals with Hb < 12 with angina, limited cardiopulmonary reserve, or other circumstances limiting their ability to carry out activities of daily living. Systematic reviews of randomized clinical trials have demonstrated an increase in thromboembolism in patients receiving these agents [53, 54]. They should be used with particular caution in patients with a previous history of thrombosis, those who have prolonged immobilization and patients with multiple myeloma who are receiving lenalidomide or thalidomide [55]. The US Food and Drug Administration (FDA) has approved a starting dose for erythropoietin of 150 U/kg three times per week or 40,000 U weekly subcutaneously, and for darbepoetin of 2.25 μg/kg weekly or 500 μg every 3 weeks administered subcutaneously [47]. US Food and Drug Administration approved labeling should be followed for dose escalation and doses should be withheld when the hemoglobin exceeds 12 g/dL until it reaches 11 g/dL where it should be restarted at a lower dose [48]. Dose reduction should occur when the hemoglobin exceeds 11 g/dL or increases 1 g/dL in a 2 week period. It is important to note that the use of ESAs in cancer patients with anemia not receiving myelosuppressive chemotherapy is not recommended. The one exception to this is the

use of ESAs to treat anemia due to low-risk myelodysplastic syndrome.

The use of ESAs in cancer patients has been implicated in both preclinical and clinical studies as a cause of tumor progression and increased mortality [56]. Given the reported risk of cancer progression and thrombosis, the safety of ESAs including Epogen, Procrit, and Aranesp are now closely monitored. The FDA requires all ESAs to be prescribed under a risk evaluation and mitigation strategy (REMS). Patients must now be told of the risks and benefits and provided with a medication guide that explains these to them. Prior to starting ESAs patients are required to sign an acknowledgement form indicating that they have been counseled on the risks and benefits of ESAs. Healthcare professionals that prescribe ESAs must complete training under the ESA APPRISE (Assisting Providers and Cancer Patients with Risk Information for the Safe use of ESAs).

Thrombopoietin Receptor Agonists

Thrombopoietin supports the proliferation and survival of megakaryocytes. More than 50% is normally produced in the liver but thrombopoietin is also produced in the kidney, skeletal muscle, and marrow stroma [57]. Receptors (c-Mpl) on megakaryocytes and platelets bind it and remove it from the circulation [58]. Elimination of the genes for thrombopoietin or for its receptor reduces platelet numbers by 90% [59]. Thrombopoietin also primes platelets to aggregate by activating the phosphoinositol 3-kinase signaling pathway [60] and supports the proliferation of hematopoietic stem cells [61].

When truncated recombinant thrombopoietin (megakaryocyte growth and development factor) modified with polyethylene glycol was administered subcutaneously to platelet donors, it stimulated antibody production which cross-reacted with endogenous thrombopoietin in some donors, resulting in severe thrombocytopenia [62]. This adverse occurrence led to a search for molecules which would bind and stimulate the thrombopoietin receptor without stimulating potentially dangerous antibodies to thrombopoietin [45]. Although such molecules are not true HGFs, such agents are included here because of their potential clinical impact.

Romiplostim (NPlate) is a peptibody with four copies of a thrombopoietin receptor-binding peptide on an immunoglobulin scaffold [45]. It increases platelet counts in healthy individuals and in those with refractory ITP [63, 64] where it has been approved for treatment. Its safety and effectiveness have been demonstrated in patients with lower-risk MDS [65, 66]. However the manufacturer clearly states that it is not indicated for the treatment of thrombocytopenia due to MDS or other causes other than chronic ITP. There are reports of an increase in blast cell counts and progression to AML with the use of romiplostim in patients with MDS. The starting dose is 1 mcg/kg once weekly subcutaneously. The dose should be adjusted in weekly increments of 1 mcg/kg to achieve and maintain a platelet count >50 K/μL. The maximum weekly dose is 10 mcg/kg. If counts do not increase 4 weeks after the maximum dose then further doses should be withheld.

Eltrombopag (Promacta) is a small molecule agonist of the thrombopoietin receptor c-mpl [67]. It is approved for the treatment of chronic ITP in patients who have not had an adequate response to treatment with steroids, intravenous immunoglobulin, and splenectomy. The starting dose is 50 mg daily orally. Care should be taken in patients with hepatic dysfunction where the dose should be lowered to 25 mg daily. Eltrombopag carries a boxed warning for hepatotoxicity and transaminase levels must be measured prior to starting and closely thereafter. Eltrombopag is also effective in improving hematopoiesis in patients with aplastic anemia [68]. In a phase 2 study of 25 patients with severe aplastic anemia, eltrombopag at a starting dose of 50 mg daily for 12 weeks (the dose could be escalated to a maximum of 150 mg daily to achieve a response), 44% of patients had a hematologic response with 36% achieving platelet transfusion independence.

Non-Hodgkin and Hodgkin Lymphoma

Use of Myeloid Growth Factors in Patients with Febrile Neutropenia

Factors associated with an increased risk of mortality in conjunction with neutropenic infection are listed in Table 58.1. Older patients clearly have an increased risk of mortality from neutropenic infection [69–71]. A Spanish study which included patients with lymphoma (and solid tumors) showed that among patients with febrile neutropenia following chemotherapy who had one or more high-risk factors, those randomized to receive G-CSF (5 μg/kg daily) had a shorter duration of grade 4 neutropenia and antibiotics (median 1 day) and a 2 day shorter hospital stay [72]. These data, along with ASCO and NCCN guidelines [22, 23], support

Table 58.1 High-risk factors in patients with neutropenic fever

1.Expected neutropenia $<0.1 \times 10^9$/L
2.Age > 65
3.Poorly controlled lymphoma
4.Pneumonia or other clinically documented infections
5. Hypotension with multiorgan dysfunction
6.Invasive fungal infection
7.Hospitalization at time of fever development
8.Neutropenia expected to last >10 days
9.Prior episode of febrile neutropenia

the use of myeloid growth factors in patients with Hodgkin or non-Hodgkin lymphoma (NHL) and risk factors for excess mortality who develop neutropenic fever.

Prophylactic Use of Myeloid Growth Factors

In individuals with lymphoma, the risk of neutropenia following chemotherapy increases with age [73–75]. A retrospective study in patients with NHL reported a 34% incidence of neutropenic fever in those over 65 compared to 21% in younger patients. The duration of neutropenia was a median of 4 days longer in the older patients [76].

The 2006 Update Committee of the American Society of Clinical Oncology agreed unanimously that prophylactic administration of myeloid growth factors is justified following chemotherapy whenever the expected rate of febrile neutropenia is 20% [22]. This recommendation was based on the inclusion of reduced toxicity and improvement in quality of life among clinical outcomes that may justify the use of a drug. The committee recommended the use of prophylactic CSF for patients with diffuse aggressive lymphoma 65 years and older treated with CHOP or more aggressive regimens to reduce the risk of febrile neutropenia and infection.

Select regimens for treating high grade lymphomas have utilized neutropenia as a biomarker to inform proper dose adjustment for subsequent cycles. Dose adjusted rituxan, etoposide, prednisone, vincristine, cyclophosphamide, and doxorubicin (dose adjusted REPOCH), a regimen being more widely utilized in NHL, utilizes standard prophylactic CSFs following chemotherapy administration with subsequent monitoring of resultant neutropenia [77]. The degree of neutropenia that occurs following each cycle dictates the dosing of chemotherapy for the following cycle. With the support of CSFs, chemotherapy dosing can be maintained or increased in patients who do not develop severe neutropenia.

In many clinical settings, the usefulness of prophylactic CSFs is uncertain. A meta-analysis of 12 randomized trials, including 1823 patients with NHL and Hodgkins disease, found a significantly reduced risk of febrile neutropenia with prophylactic myeloid growth factors but no reduction in the number of patients requiring intravenous antibiotics, infection-related mortality, or survival [78]. Furthermore, although the CSFs are effective in reducing the risk of febrile neutropenia when its risk is 20% [79, 80], questions over cost-effectiveness are largely responsible for wide variations in clinical practice. Although cost-effectiveness is difficult to precisely determine, inclusion of indirect costs of febrile neutropenia, e.g., loss of productivity, may make their administration cost-neutral in some settings where more direct calculations are less favorable [81].

Table 58.2 High-risk factors for development of neutropenic fever

1.Prior chemotherapy or radiation therapy
2.Persistent neutropenia
3.Bone marrow involvement by tumor
4.Recent surgery and/or open wounds or other clinically documented infections
5.Liver dysfunction (bilirubin >2.0)
6.Renal dysfunction (creatinine clearance <50)
7.HIV positive

Aside from elderly patients with NHL receiving aggressive chemotherapy, the use of prophylactic CSFs in patients with Hodgkin or NHL should be based on individual patient risk factors. Patients with Hodgkin or NHL who experience neutropenic fever with chemotherapy not accompanied by growth factor prophylaxis should receive prophylaxis with subsequent doses [22]. In patients who do not receive prophylactic CSFs who develop fever, growth factors should be administered to patients at high risk for complications or who exhibit other characteristics predictive of poor clinical outcome. These characteristics, based on the NCCN guidelines, are summarized in Table 58.2 [82].

Myelodysplastic Syndromes

MDS is associated with varying degrees of anemia, neutropenia, and thrombocytopenia. HGFs have been effective in improving cytopenias and quality of life, but most trials of their effectiveness have been small, non-randomized, and included heterogeneous patient populations. The studies also used disparate definitions of response. Standardization of disease classification and response to therapy is required to define the usefulness of growth factors in MDS.

A pooled analysis of more than 2500 patients with low-risk MDS (defined as less than 5% marrow blasts or an International Prognostic Scoring System (IPSS) score ≤ 1) from 162 different reports compared patients with similar baseline characteristics who received therapy with growth factors versus other therapies [83]. Almost all had one or more cytopenias prior to treatment. Patients treated with HGFs had significantly higher response rates (39.5% vs. 31.4%, $P = 0.016$) than those receiving other therapies. Responses were predominantly erythroid. Those receiving erythropoietin with or without G or GM-CSF had the highest response rates. Significant advantages in survival and progression-free survival were experienced by the growth factor group, even when controlling for all known predictors of outcome.

These data support the usefulness of erythropoietin in patients with low-risk MDS. Some studies have indicated that the combination of erythropoietin plus G-CSF may

improve erythroid response and survival in patients receiving minimal or no RBC transfusions [84], but other data do not support the value of adding a CSF to erythropoietin [83].

Once they begin to require red cell transfusions, it is reasonable to initiate erythropoietin or darbepoetin in low-risk patients. Patients with pre-transfusion erythropoietin levels below 100 mU/L and whose transfusion needs are minimal are most likely to respond [85]. Approximately 40% of patients respond, but eventually develop resistance. Treatment should be limited to patients whose hemoglobin is less than 10 g/dL and stopped once the hemoglobin reaches 12 g/dL. G or GM-CSF should be considered in patients with neutropenia who develop recurrent infections; however, their routine long-term use in neutropenic MDS patients is not recommended.

Thrombocytopenia is an independent adverse prognostic factor in MDS [86] and clinically significant thrombocytopenia occurs in more than 40% of patients [87, 88]. The only effective treatment of severe thrombocytopenia is platelet transfusion, which may be associated with acute adverse effects including febrile transfusion reactions, transfusion-related acute lung injury, and alloimmunization, which results in loss of platelet transfusion effectiveness. Multicenter studies found that weekly subcutaneous doses of the Fc-fusion protein romiplostim were well tolerated in patients with lower-risk MDS and thrombocytopenia and that 57% of patients had a complete response [65, 66]. A systematic review and meta-analysis of the use of TPO-receptor agonists in MDS suggests that Romiplastem can decrease bleeding events and platelet transfusion, but that most of the bleeding events were not clinically important and platelet transfusion reduction was relevant mainly for those receiving prophylactic transfusions. While the risk of AML progression was not increased, these data were not adequate to dismiss this risk [89].

Acute Myeloid Leukemia

Myeloid Growth Factors Following Induction

The capacity of myeloid growth factors to shorten the duration of neutropenia following induction therapy in acute myeloid leukemia (AML has been extensively studied for three decades. In order to achieve cure of AML, induction, and post-remission chemotherapy must eradicate leukemia-initiating cells as well as the bulk of leukemia cells [90, 91]. Toxicity to normal hematopoietic stem and progenitor cells, which closely resemble leukemia-initiating cells, as well as to more mature hematopoietic cells, results in prolonged marrow aplasia and frequent infections with bacteria and fungi. Approximately 10% of adult patients less than 60 and 20% of patients older than 60 die from infections after receiving standard induction therapy [22, 92], however treatment related mortality has been more recently reduced to less than 5% [93]. By reducing the duration of neutropenia, myeloid growth factors could decrease the incidence and severity of bacterial and fungal infections and mortality in patients with AML.

Numerous prospective randomized studies using G- [26, 94–98] or GM-CSF [99–103] following induction chemotherapy have demonstrated a significant reduction in the duration of neutropenia (by as much as 1 week), a shorter duration of infection, less use of antibiotics, and, in some, shorter hospitalization. Cost analyses have demonstrated conflicting results [104, 105]. Although some studies demonstrated higher rates of complete remission (CR) in patients given myeloid growth factors, a significant benefit in survival was achieved only in the Eastern Cooperative Oncology Group trial of patients aged 55–70 [102]. The lower than usual median survival of the placebo control group in that trial (4.8 months) has limited its persuasiveness [93].

Myeloid Growth Factors Following Intensive Post-remission Chemotherapy

Results similar to those following induction therapy have been reported in a randomized trial of G-CSF following post-remission therapy in patients with AML, with shortened durations of neutropenia (by 1 week), antibiotics, and hospitalization [106]. The incidence of documented infections, toxic death, disease-free, and overall survival, however, was not affected.

Growth Factor Priming

Demonstration that myeloid leukemia cells express myeloid growth factor receptors on their surface and that growth factors could stimulate differentiation of leukemic progenitors led to generally unsuccessful attempts to induce differentiation of leukemic cells in selected patients. Greater interest was generated by the demonstration that GM and G-CSF increased the proportion of leukemia cells in S phase [107–109] and that their use in combination with cytarabine increased incorporation of cytarabine into leukemia cell DNA and improved cytotoxicity [107–109]. Despite this evidence that growth factors recruit leukemic cells into S-phase and improvement in DFS in some studies of growth factor priming, randomized trials have not shown a significant benefit in survival [110–115]. The lack of clinical benefit may result from the modest increase in blasts recruited into S phase (6.0–10.7% in one study [109]).

Lowenberg et al. reported results of a trial of 640 adults ≤60 years of age randomized to receive or not receive G-CSF

starting the day before cytarabine administration in each of two induction courses [113]. Although CR rates were not significantly different, the group receiving growth factors experienced a significantly lower relapse rate and better DFS, but without improved survival. Patients with standard risk cytogenetics who received growth factor priming showed improved survival due to reduced relapse rates. A French trial in young adults (<50 years), which administered GM-CSF with induction and post-remission chemotherapy, demonstrated significantly higher CR rates and a strong, but not significant, trend toward better EFS in growth factor primed patients [114]. Patients with intermediate risk AML had significantly better EFS using growth factor. Despite the tantalizing suggestion that priming might improve the effectiveness of induction chemotherapy, the entirety of results does not support its usefulness.

The chemokine receptor CXCR4 is expressed on leukemia blasts, as it is on normal hematopoietic stem cells and is essential for homing to the marrow. Plerixafor blocks CXCR 4/CXCL12 interactions and stromal HSC and stromal leukemia cell interactions, leading to "mobilization" of HSCs and leukemic cells from the marrow [116] into the bloodstream where they are more susceptible to chemotherapy. Combining plerixafor with chemotherapy appears to sensitize patients with relapsed or refractory AML [117]. G-CSF and plerixafor have been used to sensitize leukemic cells prior to allogeneic transplantation with preferential mobilization of leukemic cells compared to normal white blood cells, but without improving relapse rates [118]. Better mechanisms of mobilization of leukemia cells could be translated into meaningful therapeutic advances.

Potential of Myeloid Growth Factors to Cause Adverse Effects on Leukemia

In addition to recruiting leukemia cells into S phase, myeloid growth factors can also result in leukemia cell expansion [109]. This has raised concern that growth factor administration might adversely affect remission and relapse rates. Many studies in AML thus delayed growth factor administration until a marrow examination performed 10 or more days after induction demonstrated aplasia. No specific clinical evidence is available to support this approach and the virtual certainty of relapse if additional chemotherapy is not given provides compelling evidence that residual leukemic cells are present in patients with "aplastic" marrows.

In general the fear of an adverse effect on leukemia cells has not been supported by clinical trials; however, one recent study has raised concerns. Increased expression of the Type IV CSF3R isoform leading to an imbalanced ratio of Type IV to Type I expression has been reported in some patients with AML. Recent results from the prospective multicenter Acute Myeloid Leukemia Berlin–Frankfurt–Muenster (AML-BFM) 98 study randomly testing the ability of G-CSF to reduce infectious complications and outcomes in children and adolescents with AML reported a trend toward an increased incidence of relapse in the standard-risk group after G-CSF treatment. Patients overexpressing CSF3R isoform IV randomly assigned to receive G-CSF after induction showed a 5-year cumulative incidence of relapse of 50% ± 13% compared to 14% ± 10% with low level isoform IV expression ($P = 0.04$). The level of isoform IV expression had no significant effect in patients not receiving G-CSF [119].

While some guidelines have approved CSF use following induction therapy, most experts do not advocate their routine use in that setting [93]. They are used by some clinicians in older patients. The use of prophylactic growth factors is not recommended in refractory or relapsed AML.

Although their administration during intensive post-remission chemotherapy has not been demonstrated to improve survival, their use is recommended by the Update Committee for their potential to derail infections and hospitalizations. These benefits must be balanced with the absence of improvement in other significant outcomes, questionable cost-effectiveness, and potential for leukemic cell expansion. For patients in whom myeloid growth factors are not given prophylactically, they may be useful in the setting of severe neutropenia and life-threatening infection.

Acute Lymphocytic Leukemia

Several studies of myeloid growth factor support have demonstrated a reduced duration of neutropenia following remission induction and post-remission chemotherapy in ALL [120–126]. A CALGB trial demonstrated shorter duration of neutropenia following induction (by a median of 6 days), significantly shorter hospitalizations, fewer deaths with induction, and a higher, though not significantly, rate of CR in patients receiving myeloid growth factors. Although growth factors were associated with faster neutrophil recovery following each of two post-remission treatments (by 9 and 6 days respectively), patients did not complete the first three rounds of therapy more quickly. Survival and DFS were not improved [120].

Dose and timing have been analyzed in this setting. A dose of 5 mcg/kg/day of G-CSF during induction significantly shortened the neutropenic period compared to a dose of 2 mcg/kg/day [125]. Administration of growth factors early (within 1 week) after initiation of chemotherapy, compared to late (10–12 days after initiation of therapy) results in faster neutrophil recovery [121], less antibiotic and antifungal use [127], and earlier discharge (by a median of 2 days) [121], but more growth factor administered and an

uncertain impact on cost. Concerns regarding expansion of leukemic clones exist, but less so than in AML. Based on evidence indicating neutropenic days and hospitalization are shortened and that severe infections and induction deaths can be decreased, growth factors are used routinely following intensive induction and post-remission therapy in ALL.

Use of HGFs in Other Hematologic Malignancies

The ASCO guidelines for myeloid growth factors and erythropoietin apply to patients with multiple myeloma, chronic lymphocytic leukemia (CLL), and low-grade lymphomas. The committee now cautions against the use of erythropoiesis-stimulating agents (ESAs) under any circumstances given established increased risks associated with ESAs, including increased venous thromboembolism, stroke, tumor progression, and mortality [128]. In both chronic myeloid leukemia and CLL, severe neutropenia in patients receiving tyrosine kinase inhibitors is generally best handled by withholding drug, rather than with the use of myeloid growth factors. In contrast, patients with CLL receiving aggressive fludarabine containing regimens associated with a high incidence of neutropenia appear to benefit from G-CSF prophylaxis [129].

Growth Factors in Autologous Transplantation

Autologous hematopoietic stem-cell transplantation is an effective therapy for many hematologic malignancies, particularly Hodgkin and NHL and multiple myeloma. It requires collection of hematopoietic stem cells which rapidly produce mature hematologic cells following transplantation. Although unstimulated marrow or blood can serve as a source of these cells, "mobilized" peripheral blood is a much better source and is standard.

Animal studies indicate that hematopoietic stem cells, rather than more differentiated progenitors, give rise to early hematopoietic reconstitution following transplantation [130]. Quantification of CD34+ cells in the collected product serves as a surrogate of stem cell number and the number of CD34+ cells infused reliably predicts the speed of hematologic recovery after transplantation [131–133]. The number of CD34+ cells in the bloodstream is increased several-fold by the myeloid growth factors [134–136]. G-CSF is significantly more effective than GM-CSF in mobilization of CD34+ cells [135–138], and has been utilized alone, or in combination with non-stem-cell-toxic chemotherapeutic agents (e.g., cyclophosphamide [136] or etoposide [132]), combination chemotherapy, e.g., ifosfamide, cisplatin, etoposide (ICE) [139], or more recently with plerixafor [140, 141], a novel small molecule which inhibits the marrow binding of stromal derived factor 1 α (SDF-1α) to the CXCR4 chemokine receptor expressed on CD34+ cells.

Most centers target a dose of 4–5 × 10^6 CD34+ cells/kg, but doses ≥2 × 10^6 are generally adequate for transplantation [142]. Lower doses often result in delayed or inadequate hematopoietic recovery [143]. CD34+ cells are collected by aphereses. Collection within one or two procedures minimizes machine and personnel time, expense and inconvenience [144, 145], but in a large multi-institute study, only 20% of patients with NHL receiving G-CSF alone achieved CD34+ cell doses ≥5 × 10^6/kg within 4 apheresis days [140]. More that 20% of patients are poor mobilizers, defined by the need for more than four aphereses or additional mobilization attempts [143–145]. Extensive previous chemotherapy, particularly with stem-cell-toxic agents, prior radiation, female sex, high body mass index, marrow involvement with malignancy, and thrombocytopenia preceding mobilization are associated with poor mobilization [142, 143]. Poor mobilization is associated with poor survival [143]. In contrast, patients who are supermobilizers, i.e., collect ≥8 × 10^6 CD34+ cells/kg, have improved survival [146]. It is unclear whether the prognostic value of cell dose may be a surrogate for better general health and other good risk factors, although it was found to an independent favorable prognostic factor in multivariate analysis which included known prognostic factors [146].

When G-CSF, the most commonly used mobilization agent, is administered alone, it is generally given at a dose of 10 μg/kg subcutaneous daily for 4 days prior to and then throughout the duration of apheresis. Similar doses can be given following chemotherapy. Although chemotherapy combined with G-CSF is more effective in mobilizing CD34+ cells [136], this is balanced by a 20–30% incidence of neutropenic fever and other chemotherapy toxicities [132, 136]. Further, the appropriate day for starting apheresis is unpredictable after chemotherapy-based mobilization, complicating scheduling. Chemotherapy should be utilized for mobilization only when it is integrated appropriately into the treatment plan, e.g., a planned course of ICE in Hodgkin and NHL. Similarly, high dose etoposide appears to be as effective as standard salvage combination therapy regimens in obtaining objective response in Hodgkin and NHL [132]. In individuals who have achieved a CR to combination therapy, additional chemotherapy may not be beneficial and its use solely for efficient mobilization is not justified. In patients who undergo chemotherapy plus G-CSF, once the WBC rises from its nadir, peripheral blood CD34+ counts are useful in determining when apheresis should be initiated [133, 147, 148].

Because only a small proportion of lymphoma patients procure targeted CD34+ cell numbers with four aphereses, and because of the disadvantages of chemotherapy, plerixafor use in combination with G-CSF has grown tremendously. Its effectiveness was demonstrated in placebo-controlled,

randomized trials in myeloma [141] and NHL [136]. In NHL, its addition to plerixafor improved the percentage of patients procuring ≥5 × 10^6 CD34+ cells within four apheresis days from 20 to 59% [140]. In myeloma, procurement of ≥6 × 10^6 CD34+ cells with 2 days was improved from 34.4 to 71.6% [130]. The combination should be routinely utilized in patients considered at high risk of failure to collect adequate numbers of CD34+ cells. The considerable expense of plerixafor is balanced, at least in part, by the need for fewer aphereses and the associated expense, risk, and inconvenience of numerous aphereses and remobilization attempts [144, 145]. G-CSF is also administered at most centers following autologous transplantation where it modestly accelerates neutrophil recovery and shortens hospitalization [149]. A G-CSF regimen identical to that used for autologous stem-cell mobilization is also the standard mobilization method for peripheral blood donors for allogeneic transplantation. Aside from bone-pain, it is associated with few side effects and a vast majority of healthy donors mobilize adequate numbers of cells within two apheresis procedures.

The use of mobilized peripheral blood cells for allotransplantation is associated with an increased incidence and duration of chronic graft-versus-host disease compared to bone marrow (presumably because of a higher number of T cells) but with accelerated neutrophil and platelet recovery [150, 151]. Allogeneic recipients of bone marrow who receive G-CSF following transplant appear to experience higher incidences of acute and chronic graft-versus-host disease [152].

Delayed erythroid recovery occurs commonly following transplantation. Some patients require red cell transfusions for up to a year. While multiple factors contribute, prolonged anemia following both allogeneic and autologous transplantation is associated with an inadequate erythropoietin response [153]. Administration of erythropoietin appears to modestly decrease the transfusion requirement after allogeneic, but not autologous transplantation [154], although conflicting results have been reported [155]. Most centers do not routinely use erythropoietin following transplantation, but it may be beneficial in selected patients with delayed erythroid recovery.

Eltrombag has proven effective in elevating platelet counts in patients with persistent thrombocytopenia following autologous and allogeneic transplantation [156]. Its use is reasonable in selected patients.

Can Growth Factors Cause Leukemia?

There has been considerable concern over the potential of myeloid growth factors to accelerate leukemic expansion in patients with AML, as has been discussed, or to cause leukemia in some individuals. Two separate studies of over 5000 individuals failed to detect any increase in AML in peripheral blood stem-cell donors receiving G-CSF [157, 158]. A review of 25 randomized trials of patients receiving chemotherapy for solid tumors or lymphoma with or without G-CSF demonstrated a doubling of the incidence of MDS/AML, although all cause mortality was decreased among patients receiving G-CSF [159]. The increased risk of MDS/AML may be attributable to the increased dose intensity of leukemogenic agents which was delivered in those receiving G-CSF.

In addition to the increased incidence of AML in SCN associated with G-CSF use, a significantly increased incidence of MDS/AML was reported in Aplastic Anemia patients receiving immunosuppressive therapy and G-CSF compared to those not receiving G-CSF [34], as described earlier. A Japanese study demonstrated a strong leukemia risk in children with aplastic anemia receiving G-CSF, particularly those resistant to immunosuppressive therapy [160]. Interestingly, this group of patients had a high incidence of 7q− and monosomy 7. Fluorescence in situ hybridization (FISH) identifies monosomy 7 in many patients with marrow failure and normal cytogenetics by conventional metaphase analysis and in those in whom metaphase analysis could not be performed. Basic studies demonstrate that high concentrations of G-CSF increase the proportion of monosomy 7 cells in vitro and that CD34+ cells in MDS with monosomy 7 express increased proportions of G-CSFR isoform IV mRNA [161]. These data strongly suggest that pharmacologic doses of G-CSF increase the proportion of preexisiting monosomy 7 cells, rather than promote the malignant transformation of normal hematopoietic cells.

It is not yet clear whether G-CSF can directly contribute to the development of AML, but it has been suggested that the anti-apoptotic effect of G-CSF could rescue hematopoietic stem or progenitor cells which develop otherwise lethal mutations from chemotherapy. Rescue of these cells, which would otherwise die, might allow such cells to develop into myeloid malignancies [45].

The Future

The full benefits of HGFs have not yet been realized. Despite carefully crafted recommendations on their use, considerable variation in clinical practice exists. Concerns include their expense, inconvenience, and adverse effects. These concerns may be alleviated should ongoing basic work lead to the development of oral agents which stimulate hematopoiesis.

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Bleeding and Thrombosis in Hematologic Neoplasia

59

Joseph J. Shatzel, Robyn Scherber, and Thomas G. DeLoughery

Leukemia and the Myelodysplastic Syndromes

Coagulation Defects

While both bleeding and thrombosis are associated with multiple types of leukemia and their treatment, acute promyelocytic leukemia (APL) is the leukemia classically associated with coagulation defects with evidence of disseminated intravascular coagulation (DIC) occurring in a majority of patients. The pathogenesis and therapy of coagulation defects are discussed separately in Chap. 23.

APL aside, clinically overt DIC is evident in up to a third of patients with other forms of acute leukemia, which may be present at diagnosis or manifest shortly after beginning treatment [1]. Patients with myeloid leukemia who present with higher peripheral blast counts appear to be at higher risk perhaps because of the propensity of myelomonocyte and monocytic leukemia to present in this manner [2–4]. Within the various subtypes of myeloid leukemia, the monocytic phenotype (M5 in the FAB classification) appears to have the highest incidence of DIC, with about 25% of patients having intravascular coagulation during their care [5–7], and about 50% having evidence of coagulopathy including increased D-dimer, thrombin-antithrombin III-complexes, low fibrinogen, low α2-PI plasma complex levels, or elevated elastase-α1 antitrypsin complexes [5, 7, 8]. DIC has also been reported in chronic myelocytic leukemia (CML), both with and without blast crisis [9–11] and in acute lymphocytic leukemia, especially of the T-cell type; however, it occurs far less commonly than in acute myeloid leukemia (AML) and its variants [9, 12, 13]. Biochemical markers of DIC have been found in many patients with acute lymphoid leukemia (ALL) including elastase-α1-antitrypsin complexes and rapid fibrinogen turnover [14]. When studied prospectively, DIC was seen in 12% of patients with ALL before chemotherapy, which rose to as high as 78% during chemotherapy [15].

A study evaluating the expression of markers of coagulation in AML patients has suggested that exaggerated tissue factor expression and circulating plasma microparticles may be the triggering source of DIC once sufficient levels are achieved [16, 17]. Additional non-tissue factor pathways including the shedding of cell free DNA likely contribute as well [16, 17] in APL specifically, a combination of tissue factor, cancer procoagulant, and microparticles are all produced by the malignant clone [18]. Thrombin generation is augmented in vitro as APL cancer cell lines cells undergo apoptosis, correlating with the clinical experience that DIC initially worsens with treatment of the leukemia [15, 18, 19]. APL cells also express aberrant levels of annexin II, which greatly augments the generation of plasmin by nearly 60-fold [20]. It is unknown if these mechanisms are important in coagulopathies of other forms of leukemia.

Life-threatening hemorrhage does occasionally occur in acute leukemia. Thrombocytopenia, anemia, DIC, and other coagulation defects common to acute leukemia likely contribute to the significant incidence of bleeding. A high incidence of subdural hematomas specifically has been reported in patients with monocytic leukemia, perhaps reflective of the incidence of intravascular coagulation and leukostasis [21, 22] large series of leukemia patients as a whole report that up to 7% will develop clinically significant hemorrhage [23]. Series specific to APL note a 5% incidence of fatal hemorrhage [24]. Hemorrhagic death in APL is most often attributed to cerebral (65%) or pulmonary (35%) hemorrhage [18, 25]. APL patients with a high blast count at diagnosis are more likely to have significant bleeding [26].

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Clinically, thrombosis is also a common complication in leukemic patients during their initial hospitalization after diagnosis. A large observational study analyzing the incidence of thromboembolic disease in leukemic patients found a cumulative incidence of 19% in the first 3 months after diagnosis [27]. The risk significantly dissipates after the first 3 months. A similar pattern was found in ALL patients, but with a much lower incidence of 11% in the first 3 months. Certain chemotherapeutic protocols substantially increase the risk of thrombosis as outlined later in this chapter [15].

Subclinical intravascular clotting may be common in acute leukemia. Increased levels of fibrinopeptide A or D-dimer levels reflect activation of thrombin which promote fibrin generation and consumption of coagulations factors, while decreased levels of alpha 2-antiplasmin reflect heighten fibrinolysis which can lead to bleeding. These findings are often encountered in patients with acute leukemia without overt DIC before treatment, and can promptly rise after institution of cytotoxic chemotherapy [28–30]. Decreased levels of protein C and antithrombin III and elevated levels of thrombin-antithrombin complexes have been reported as well in patients with newly diagnosed myeloid leukemia [30]. These markers of coagulation often return to normal with achievement of remission, and in a few instances, rising levels predicted relapse [31].

Several other coagulation abnormalities have been reported in leukemia, including low factor V levels in Philadelphia chromosome-positive CML in children [32], and a decrease in the thrombin-sensitive subunit of factor XIII in a patient with acute leukemia and DIC [32, 33]. One report documented the secretion of two types of plasminogen activators by human myeloid leukemia cells in vitro [34]. The quantity or type of plasminogen activator that was secreted by these leukemic cells could not be correlated with either bleeding or the clinical severity of the disease. Another study suggested that leukemic cells that lacked cell-associated procoagulant activity can still incite a coagulopathy via the release of IL-1, a potent stimulator of endothelial tissue factor synthesis and inhibitor of thrombomodulin, an essential cofactor for the activation of protein C [35].

The most effective method to treat DIC is to treat the underlying leukemia but one must be prepared for an exacerbation of the DIC when tumor lysis occurs. The basic tests of coagulation should be obtained at least daily and more often if potentially clinically significant coagulation defects develop, using the results of these test guides one in replacement therapy of DIC (Table 59.1). Past concerns about “feeding the fires” have not been shown to be clinically valid and one should strive to maintain hemostatic competence to avoid severe bleeding [36–38]. Keeping the fibrinogen level over 150 mg/dL via the use of cryoprecipitate transfusions is reasonable in the leukemic patient with DIC [39]. Plasma replacement may be needed to correct multiple factor deficiencies [40]. A practical goal is to decrease the aPTT to less than 1.5 times normal if possible [39]. Heparin therapy is rarely used for the leukemia patient with DIC, and there is little evidence suggesting it offers a benefit. In APL specifically, early initiation of all trans retinoic acid (ATRA) is imperative to decrease bleeding risks, and should be considered even before molecular confirmation if APL is suspected [41]. Many authors advocate for platelet transfusion early in APL to increase levels >50,000/μL to prevent bleeding [39]. Some have suggested recombinant human soluble thrombomodulin as a potential treatment option, although current studies are limited to in vitro analysis [18].

Table 59.1 Management of disseminated intravascular coagulation

Tests to perform to assess hemostasis
1. Hematocrit
2. Platelet count
3. Prothrombin time
4. Activated partial thromboplastin time
5. Fibrinogen level
Therapy based on test results
(a) Platelets <50–75,000/μL—Give platelet concentrates or 6–8 pack of single donor platelets
(b) Fibrinogen <150mg/dL—Give 10 units of cryoprecipitate
(c) Hematocrit below 30%—Give red cells
(d) Protime >INR 2.0 and aPTT abnormal give 2–4 units of FFP

Platelet Defects

Studies of platelet function in patients with acute leukemia have been difficult because severe thrombocytopenia is often present. However, platelets from patients with acute myelocytic leukemia show abnormalities in size and granulation on light microscopy, as well as marked variations in granular content, disturbances of microtubule function, and striking dilatation of the open canalicular system by electron microscopy [42, 43]. Flow cytometry studies can show a population of platelets with loss of platelet activation markers [44]. Some investigators believe that they can distinguish two populations of cells—a normal population and a second “leukemic” population, showing marked variations in size, shape and granular structure [43].

Platelet function in acute leukemia has been shown to be abnormal, with defects of platelet aggregation in response to epinephrine, ADP, and collagen, as well as a lack of storage granules and impaired platelet factor III release [42, 45–47]. Patients with AML have significantly less in vivo platelet activation than patients with immune thrombocytopenic purpura (ITP) for instance [48]. Platelet function and morphology usually return to normal after a complete remission has been attained. The etiology of the platelet dysfunction has not been clearly defined but is most likely attributable to disease-induced abnormalities in the megakaryocyte. Alternatively, some platelets may undergo the release reaction within the circulation and thereby have deficient storage pool ADP and other morphologic changes [42].

Studies of platelet morphology and function in the various myelodysplastic syndromes (MDS) have found abnormalities similar to those reported in acute leukemia and the chronic myeloproliferative diseases. In many cases, megakaryocytes are normal to moderately increased in number in the marrow, are quite small, and have decreased nuclear "ploidy" with abnormal cytoplasmic granulation [43, 49]. As in acute leukemia, the platelets show numerous changes on electron microscopy, including unusual giant granules, lack of microtubules, and a dilated open canalicular system [42, 50]. A variety of platelet function defects have been described, but impaired platelet aggregation and a variably increased bleeding time are most prevalent [51, 52]. In one study 75% of MDS patients demonstrated reduced platelet aggregation with epinephrine, followed by decreased PA with arachidonic acid (54%), ADP (46%), collagen (43%), and ristocetin (22%) [53]. Abnormalities of platelet function occur in acute megakaryocytic leukemia including defects in platelet aggregation ex vivo but these are poorly related to clinical bleeding [54–58]. Platelet dysfunction and thrombocytosis has also been reported in refractory anemias or MDS associated with deletion of chromosome 5q or chromosome 3 [59–61].

Hairy Cell Leukemia

Hairy cell leukemia can lead to both quantitative and qualitative platelet defects. The platelet count in hairy cell leukemia is usually low, due to marrow infiltration with lymphocytes, or hypersplenism and the platelets often show abnormal aggregation to epinephrine, ADP, and sometimes collagen [62–64]. Furthermore, the granular content of ADP and serotonin is decreased, the release of ADP may be impaired, and prostaglandin synthesis may be inhibited [65]. On electron microscopy, the platelets are virtually agranular and show a dilated open canalicular system [65]. Several reports indicate that most of the platelet function defects and abnormal morphology return to normal after splenectomy [63, 65]. The mechanisms for these changes are unknown, but it has been postulated that these platelets undergo activation in the spleen and then recirculate with an acquired storage pool deficiency (i.e., "exhausted" platelets) [65]. While rates of bleeding associated with these platelet defects are not well described, spontaneous splenic ruptures have been rarely reported [66, 67].

Myeloproliferative Neoplasms

The myeloproliferative neoplasms (MPNs) which includes essential thrombocythemia (ET), polycythemia vera (PV), primary myelofibrosis (PMF), (previously known as agnogenic myeloid metaplasia or chronic idiopathic myelofibrosis), and chronic myelocytic leukemia (CML) are clonal disorders characterized by an expansion of the myeloid precursors in the marrow and blood, along with splenomegaly. All of these disorders are associated with thrombocytosis, and, with the notable exception of CML, commonly provoke bleeding or thrombosis. The diagnosis of PV and many cases of ET can now be made much earlier with the advent of the testing for common genetic mutations including JAK2, Calreticulin (CALR) and of the thrombopoietin receptor (myeloproliferative leukemia, MPL). The most common JAK2 mutation is *JAK2*V617F, which leads to the deregulated and constitutive activation of a tyrosine kinase that promotes myeloid precursor proliferation. This mutation is present in approximately 98% of PV patients, 50% of ET patients, and 60% of MF patients. CALR is a calcium binding protein which has been found to induce thrombopoietin-independent growth when mutated and induce downstream *JAK2* pathway activation [68]. CALR is present in approximately 30% of ET and MF patients and *MPL* in 3–7% of ET and MF patients, respectively. The diagnosis of PV and ET was previously made via endogenous erythroid colony assays, but now can be readily secured via peripheral blood or bone marrow genetic testing [69–71]. For patients who do not have a clonal marker of their MPN ("triple negative" MPN), there are clinical criteria that allow for diagnosis [72].

Bleeding and Thrombosis

Excessive bleeding occurs frequently in patients with PV, ET, and PMF and is usually due to both qualitative and quantitative abnormalities in platelet function and number [73, 74]. The incidence of hemorrhage is difficult to quantitate because the frequency can vary relative to the underlying disease process, stage of disease, age, and the presence of comorbid conditions. Hemorrhage is more common in PMF, which is felt to be a result of the thrombocytopenia, qualitative platelet defects, or even DIC [75]. Overall, about 25–30% of patients will have bleeding, in 5% it will be severe, but it will be fatal in less than 1% [76–84]. Symptoms are usually limited to easy bruisability or epistaxis, but gastrointestinal bleeding seems particularly common in PV.

Thrombosis occurs more frequently in PV and ET than in CML, and is a major cause of morbidity and mortality in patients with myeloproliferative neoplasm. Accordingly, reducing thrombotic complications associated with disease remains central therapeutic goal when considering treatment. A recent report highlights that thrombosis is present in 12% of ET patients evaluated, which included arterial (79/109) and venous (37/109) thrombosis [85]. As demonstrated in this and previous studies, arterial occlusion tends to be most common, followed by venous thromboembolism, and then microvascular thrombosis (digital skin necrosis, or

erythromelalgia) [73, 77, 79, 85–95]. The mechanism of underlying thrombophilia is diverse among these patients, consisting of a milieu of inflammatory cytokines, hyperviscosity, upregulation of endothelial adhesion receptors, acquired activated protein C (APC) resistance, overexpression of circulating procoagulant microparticles (MPs), and overall coagulopathic activity of the vascular endothelium. Platelets have been shown to have increased P-selectin and tissue factor [96].

The overall frequency of thrombosis is difficult to calculate, and underappreciated but it is more common than bleeding, and causes greater morbidity and mortality [74, 82]. At initial presentation, the reported incidence of thrombosis and bleeding in ET varied from 11–25% to 3.6–37%, respectively. The corresponding figures for PV were 12–39% and 1.7–20%. Factors that could have accounted for the relatively wide range of values include patient selection, definitions of events, accuracy in data reporting, and the effect of therapy. Cross-sectional studies suggest that approximately 30–40% of patients have thrombosis, and several large studies indicated that thrombotic complications occurred at approximately 3.4–8%/year [90, 94, 95, 97, 98]. The most common thromboses are cerebral vascular occlusion, deep venous thrombosis of the lower extremities, vascular occlusion of the digits, and particularly central abdominal thromboses (portal, hepatic, mesenteric vein). Increasing age, previous thrombosis, smoking, and a previous history of thrombosis predispose to thromboembolism in ET or PV [74, 99–103]. Splenomegaly has also been found to accompany a higher risk of thrombosis in ET [104]. Phlebotomy and elevated hematocrit add to thrombotic risk in PV. Guidelines now recommend a goal hematocrit less than 45% as this results in significantly lower mortality due to reduced thromboembolic events [105]. This correlation between thrombosis and elevated blood counts is not as clear for platelets. Overall, the elevated platelet count does not correlate with the risk of thrombosis for PV or ET [106, 107]. For example, some patients with very early MPN documented by molecular assays who have normal or near normal platelet counts can have repeated severe thromboses [107–110]. Others, for example, young women with platelet counts of more than 2,000,000/μL, often remain free of symptoms for many years. Several studies have showed that the majority of thrombotic complications occurred in individuals with platelet counts of less than 600,-800,000/μL suggesting factors other than platelet number account for the thrombosis risk [77, 107, 111, 112]. The role of leukocytosis as a risk factor for thrombosis remains controversial. Several studies suggest a WBC greater than 10,-15,000/μL may be a risk factor for thrombosis [99, 102] while other studies do not support this [113] or suggest it is not a risk factor for low risk patients [114].

The association between genetic mutations and thrombosis in the MPNs is still under investigation. CALR mutations, which are found almost exclusively in ET and PMF, tend on average result in lower thrombotic risk, although this correlation is most noticeable in type I (52-bp deletion) CALR mutations than in type II (5-bp TTGTC insertion) mutations [115]. ET patients with the JAK2 mutations have a double risk of thrombosis compared to non-carriers [116] and being homozygous for JAK2 mutations may further increase this risk [117–119]. The function of JAK2V617F in thrombotic risk is still under investigation, but can be attributed in part to changes in platelet function (i.e., elevated expression of p-selectin, increased platelet migratory ability, elevated erythropoietin signaling) and due to a direct effect on some non-hematopoietic cells such as liver endothelial cells in patients with Budd-Chiari [120–122].. MPL does not appear to be a risk factor for thrombosis [85]. Patients negative for mutations in JAK2, CALR, or MPL tended to have lower thrombotic risk [115].

MPN patients also have increased symptom burden compared to age-matched controls [123, 124]. Although MPNs have been found to have significant cytokine deregulation which contributes to symptom development, many symptoms can be a consequence of microvascular and large vessel thrombosis [125]. Erythromelalgia is a syndrome of painful toes and fingers associated with MPN. It is characterized by thrombocytosis and biopsy evidence of microvascular subendothelial vascular proliferation. In some cases, symptomatic platelet-mediated thromboses occur in the digits which can produce tissue ischemia and even gangrene [126, 127]. Erythromelalgia secondary to MPN often responds dramatically to low doses of aspirin [127, 128]. Headache is another common symptom that has been associated with platelet dysfunction, anemia, increased nitric oxide production, and microcirculatory disturbances. A trial of aspirin is recommended as first line treatment in MPN-associated headache. If the headache persists despite aspirin therapy, a combination of cytoreduction and aspirin is recommended [129]. Alternative symptoms that could be contributed to thrombosis include abdominal pain or discomfort (splanchnic vein thrombosis), sexual difficulties, cough, bone pain, and pruritus.

Mechanisms of Platelet Dysfunction

Sensitive tests of platelet function are frequently abnormal in chronic MPN, particularly PV, ET, and PMF. Tests of platelet reactivity correlate poorly with elevations of the platelet count or with syndromes of bleeding and thrombosis [130–133]. However, a rough relationship exists between a high circulating platelet count, impaired platelet function, and bleeding [76, 131, 134–137]. High platelet counts are also associated with increased platelet reactivity and vascular thrombosis, erythromelalgia, and peripheral gangrene [127, 128, 132, 138, 139]. Studies determining clonal status of patients with ET have demonstrated that there is a great deal

of heterogeneity in the presence of clonal disease [140]. Notably, there is clear evidence that clonal disease portends a higher risk of thrombosis [141–143].

Decreased Platelet Function

Platelet function has been examined in several series of patients with chronic MPN [51, 137, 144–152] (Table 59.2). Although abnormalities in tests of platelet function are quite common in vitro, the bleeding time is usually normal or, in about 20% of patients, mildly prolonged [137, 145, 146, 149, 151]. Rarely, the presenting clinical scenario is excessive bleeding and a markedly prolonged bleeding time, which is most often seen in patients with PMF [33]. The most common platelet function defect is abnormal platelet aggregation following the addition of collagen, ADP, or particularly epinephrine, to platelet rich plasma [137, 145, 146, 149, 151, 153, 154]. In most reports however, little correlations have been found between changes in platelet function, prolongation of the bleeding time, and clinically evident bleeding [51, 130, 147]. It should be noted that platelet aggregation testing is usually normal in patients with a reactive thrombocytosis, even if the platelet count exceeds 1,000,000/cmm [146].

A myriad of platelet function disorders have been discovered in patients with MPN. These include specific defects in the platelet membrane, the release of platelet coagulant activity, abnormal prostaglandin synthesis, and disordered platelet-dense granule composition and/or function. Platelet membrane abnormalities include the deficiency of several glycoproteins important for normal platelet aggregation [155, 156], increased numbers of platelet Fc receptors for immune complexes, or aggregated IgG [157], and prostaglandin D_2 or thromboxane A_2 receptor numbers and/or function [158].

Platelet prostaglandin synthesis has been shown to be inhibited, with decreased production of thromboxane A_2 after stimulation of the arachidonic acid pathway [155, 159]. Defects in lipoxygenase activity has been reported [160] as well as reduced platelet generation of lipoxin [161]. Finally, patients have been reported with decreased numbers of dense granules in their platelets, as visualized by electron microscopy. A reduction in dense granule content has been correlated with prolongation of the bleeding time [148, 162, 163]. Decreased alpha granule content has also been described [164, 165]. Other defects include the inability of stimulated platelets to expose procoagulant phospholipid despite normal membrane phospholipid composition [73, 137, 166].

Finally, patients with very high platelet counts (greater than one million) can have acquired von Willebrand's disease demonstrated by reduced ristocetin cofactor activity despite normal plasma levels of von Willebrand antigen and factor VIII coagulant activity [167–169]. The decreased plasma von Willebrand factor activity may be due to accelerated proteolysis of von Willebrand factor after its release from endothelial cells or uptake of the high molecular weight multimers by the increased number of platelets.

Table 59.2 Abnormalities in myeloproliferative neoplasms leading to thrombosis

Acquired activated protein C resistance
Hyperviscosity
Increase circulating microparticles
Increased platelet P-selectin and tissue factor expression
Inflammatory cytokines
Prothrombotic endothelium
Upregulation of adhesion proteins

Increased Platelet Activity

Several studies have identified platelet "hyperreactivity" in patients with MPN [138, 145, 149–151, 154, 170–173] (Table 59.3). A variety of tests to assess platelet reactivity has been used including the spontaneous platelet aggregation during ex vivo testing, increased plasma level of the platelet specific protein β-thromboglobulin, decreased circulating platelet aggregate ratios, platelet phospholipid-dependent thrombin generation, and increased platelet responsiveness to inhibitory prostaglandins. Although in some reports there appeared to be a correlation between increased platelet function and vascular thrombosis, it is not possible to predict clinical thrombotic events with the use of these assays.

Several investigators have measured levels of platelet specific proteins (platelet factor 4, β-thromboglobulin, or platelet derived growth factor) in patients with MPN. Platelet concentrations of these proteins were reduced, whereas plasma and urinary levels were increased [103, 174–177].

Table 59.3 Platelet abnormalities in myeloproliferative neoplasms

Decrease platelet function
Abnormal platelet aggregation
Abnormal prostaglandin synthesis
Decrease number and function of prostaglandin D_2 and thromboxane A_2 receptors
Decreased platelet procoagulant activity
Impaired platelet function
Increased platelet Fc receptors
Platelet dense granule function and composition
Platelet membrane defects
Increased platelet function
Increase platelet β-thromboglobulin
Increased expression of platelet activation markers
Increased platelet thrombin generation
Spontaneous platelet aggregation

These findings suggest that intravascular platelet activation occurred, which could explain platelet-mediated thrombosis or marrow fibrosis [96, 103, 175, 178]. Consistent with this idea is the findings that patients with increase expression of platelet activation markers have increased risk of thrombosis [173, 179, 180].

Other Laboratory Tests of Thrombophilia

Increase levels of homocystine have been reported in patients with MPN but have not correlated with thrombosis [181–183]. A thrombin generation assay to assess parameters of hypercoagulability in MPN patients indicates the presence of acquired APC resistance in ET and PV patients, which is likely a result of a reduction in free PS levels and that the APC-resistant phenotype is influenced by the JAK2 mutational load [184].

Given that MPN are clonal diseases it may be that other cell lines are contributing to the thrombophilia. Multiple studies have demonstrated the following parameters of blood cell and coagulation activation associated with thrombosis in the myeloproliferative neoplasms: increased percentage of platelets that expressed P-selectin, overexpression of the CD11b antigen on monocytes and neutrophils, increased platelet-neutrophil complexes, increased tissue factor after polysaccharide activation in monocytes, and increased reticulated platelets [179, 184–186]. One study demonstrated increased monocyte generation of tissue factor that was linked with elevated levels of prothrombin F1.2 fragment [187]. Another study suggested that neutrophil activation is abnormally high in MPN and is associated with signs of increase coagulation [188]. An intriguing study showed that endothelial cells from patients with visceral vein thrombosis have the JAK2 mutation which may be altering their antithrombotic properties [189]. Thus, it may be an interplay between multiple cell lines that leads to the thrombosis seen in MPN.

Clinical Laboratory Tests

Laboratory tests such as the CBC, platelet count, bleeding time, and platelet aggregation studies are frequently abnormal in patients with MPN, but unfortunately they often fail to predict bleeding or thrombosis in individual patients. However, patients with PV and markedly elevated hematocrits are at substantial risk of thrombosis, and patients with PMF or others with very high platelet counts (more than 1,000,000/cmm) and markedly prolonged bleeding times are likely to have an increased risk of bleeding [190]. Aspirin and non-COX selective nonsteroidal anti-inflammatory drugs should be used with caution in the latter instance.

The JAK2 mutation can be positive in patients with normal or near normal peripheral blood counts and bone marrow morphology, particularly those with a history of arterial or venous thrombosis of unknown cause [88, 108, 191, 192]. The assay should be considered as part of a laboratory evaluation in patients with recurrent thrombosis (particularly arterial and central abdominal venous thrombosis) who don't have other hereditary or acquired hypercoagulable states. For example, in several studies over 30–50% of patients presenting with portal vein thrombosis were found to be JAK2 positive or have other markers of MPN [108, 191–193].

Therapeutic Considerations

Hemorrhage

Major bleeding complicating MPN can usually be managed with platelet transfusions to overcome disease-induced platelet function defects. In a few patients, particularly those with very high platelet counts, platelet transfusions will be ineffective. In these instances, therapeutic platelet apheresis with acute lowering of the platelet count followed by transfusion of normal platelets may temporarily improve hemostasis. Platelet reduction on a long-term basis with hydroxyurea or other therapeutic agents may decrease symptoms of bleeding. Finally, phlebotomy in patients with PV has been reported to improve platelet function tests and to decrease bleeding in some instances [164, 165].

Venous Thrombosis

Intravenous heparin followed by warfarin is indicated for most patients with acute venous thromboembolism complicating the myeloproliferative disorders. Thrombolytic therapy should be considered in patients who have acute occlusion of the hepatic or portal veins. Long-term oral anticoagulants (INR 2–3) are usually recommended for prevention of recurrent thromboses. In a few instances, liver transplantation has been successful in treating liver failure due to Budd-Chiari syndrome [194, 195]. A key to long-term success is aggressive anticoagulation posttransplantation.

Arterial and Microvascular Thrombosis

Antiplatelet therapy, usually with aspirin, is recommended for treatment of patients with cerebral, coronary artery, or peripheral vascular thrombosis. Platelet inhibitors usually suppress the platelet hyperreactivity associated with MPN and can increase the usually shortened mean platelet survival time [196]. Low doses of aspirin (80–360 mg/day) are preferable in patients with MPN because the risk of bleeding with aspirin is dose-related.

One study using 100 mg/day appeared to be effective for preventing thrombosis without excessive bleeding as long as the platelet count was kept at under 1,000,000/μL [197]. Another study randomized 518 asymptomatic, low thrombotic risk, PV patients to treatment with low-dose aspirin (100 mg daily) versus placebo and found that aspirin significantly lowered the risk of cardiovascular death, nonfatal myocardial infarction, nonfatal stroke, and major venous thromboembolism (relative risk 0.4 [95% CI 0.18–0.91]; $P = 0.028$). Total and cardiovascular mortality were also reduced by 46% and 59%, respectively. Major bleeding was only slightly increased by aspirin (relative risk 1.6, 95% CI 0.27–9.71). However, some large population trials have indicated no increased bleeding risk with aspirin use [85]. Given these results, low-dose aspirin (75–100 mg daily) is recommended in all PV patients without history of major bleeding or gastric intolerance [198, 199]. Patients with very high platelet counts should have Von Willebrant testing before starting aspirin [200].

Microvascular disease such as erythromelalgia or digital gangrene often responds dramatically to antiplatelet therapy [197]. In addition to its antithrombotic effects, aspirin may also inhibit platelet secretion of vascular growth factors and thereby reduce chronic vascular damage [201]. There is currently no data concerning the use of newer agents such as clopidogrel but these may be worth trying if the patient is aspirin intolerant.

A few patients will develop serious recurrent thromboembolic events despite treatment with aspirin. In such cases, combined anticoagulation (INR 2–3) and antiplatelet therapy should be considered [202]. The likelihood of bleeding will be increased, but the risks might be warranted in some patients. Careful monitoring will be required, particularly in patients known to have platelet dysfunction or a history of bleeding.

An even more difficult problem is whether to lower platelet counts or to give aspirin to patients with MPN who do not have a history of thrombosis. The evidence from the literature is conflicting; some studies suggest that young patients can tolerate very high platelet counts without major thrombosis, whereas others indicate that young as well as older patients remain at risk of thrombosis and bleeding [89, 203, 204]. Platelet reduction with hydroxyurea should be considered in asymptomatic older subjects with platelet counts over 1,000,000/μL, particularly if they have atherosclerosis, risk factors for arterial disease, or symptoms of vascular ischemia [205]. Also important is controlling reversible risk factors such as smoking or elevated cholesterol [206].

In addition to pharmacologic antithrombotic measures, lowering of elevated platelet counts should be considered in patients with MPN and a history of thrombosis. Hydroxyurea (1 g daily to start) is preferable to radioactive phosphorous or alkylating agents because of a lesser risk of leukemogenesis. A platelet count of 250–450,000/μL is an appropriate target. A randomized trial of high-risk patients (age over 60 or history of thrombosis) demonstrated that using hydroxyurea to maintain the platelet count less than 600,000 μL was associated with significantly less thrombosis (3.6 vs. 24%) [207]. Reduced thrombosis is felt to possibly be due to increased production of nitric oxide in vascular cells treated with hydroxyurea [208].

Anagrelide has also been used to lower platelet counts in MPN patients [209–211]. Unlike hydroxyurea clinical trial data exists to cast doubt on the efficacy of anagrelide in preventing thrombosis in high-risk patients. One long-term study of patients did demonstrate a 20% thrombosis rate in patients taking anagrelide [212]. However, platelet counts were above 400,000/μL in all patients who had thrombosis leaving unclear whether the agent would have been more successful at preventing thrombosis if the platelet counts were lower [213]. A more recent study compared hydroxyurea plus low-dose aspirin (100 mg daily) with anagrelide plus low-dose aspirin in 809 high-risk patients. Patients randomized to hydroxyurea and aspirin less likely experience major arterial or venous thrombosis, major hemorrhage, or death from a vascular cause ($P = 0.03$) [214].Additionally, anagrelide has also been associated with cardiovascular side effects and is not recommend for patients with a history of heart disease [212, 213]. Thus, hydroxyurea remains the first choice for lower platelet counts in high-risk patients.

Phase II studies have demonstrated that α-Interferon is effective in thrombophilic states, but its antithrombotic effect is unknown; moreover, its attendant side effect profile renders it difficult to utilize in the majority of patients [215–218].

Lastly, JAK2 inhibitors are progressively becoming a mainstay of therapy in the MPNs. Ruxolitinib, a JAK1/2 inhibitor, is currently FDA approved in the United States for the treatment of intermediate-2 and high-risk MF and PV patients who are intolerant or resistant to hydroxyurea [199, 219]. In a landmark study of ruxolitinib among PV patients, patients receiving ruxolitinib were noted to have fewer thrombotic events [220]. Further meta-analyses among MF and PV patients have indicated that there is indeed reduced thrombotic risk among ruxolitinib treated patients compared to patients receiving standard therapy [risk ratio 0.45, 95% confidence interval (CI) 0.23–0.88] [221]. One month following JAK2 inhibitors treatment, changes in thrombotic markers have been observed including decreased monocyte tissue factor expression, platelet monocyte aggregates, and neutrophil activation as well as increased monocyte count [222]. JAK2 inhibitors also have been found to reduce splenomegaly, which may also convey a reduction in MPN-associated SVT with portal hypertension along with the related sequelae of GI bleeding and thrombotic events.

Pregnancy

Although PV is rare to arise in pregnancy, an increasing challenge is the increase in number of young women with ET who become pregnant as they do tend to have a higher risk of

pregnancy-related complications, especially if they harbor the JAK2 mutation [223]. Interestingly, the platelet count often decreases during pregnancy, often to near normal levels lessening the need for cytoreductive therapy [224, 225]. The most common adverse event is an increase in miscarriages due to placental infarction [225–227]. Additionally, ET and PV are associated with increased risk of preeclampsia, fetal growth restriction, and maternal thrombosis and bleeding [228]. Due to the physiologic decrease in platelet count during pregnancy and concerns about fetal health, cytoreduction therapy should be avoided if possible. Most data now supports the use of aspirin throughout the pregnancy to prevent miscarriages and thrombosis. Women with a previous history of thrombosis should be therapeutically anticoagulated with low molecular weight heparin to prevent recurrence. If platelet lowering is required, interferon is the safest option but there is increasing data from sickle cell patients that hydroxyurea can be safely use starting in the second trimester [225, 229–232].

Paroxysmal Nocturnal Hemoglobinuria (PNH)

PNH is a clonal hematopoietic disorder characterized by complement mediated hemolysis and often some degree of bone marrow dysfunction [233]. The leading cause of morbidity and mortality in PNH is thrombosis, which some have described as the most vicious acquired thrombotic state in medicine [234–236]. Thrombosis accounts for 40–67% of the mortality in PNH [236]. 29 to 44% of patient will have a thrombosis during the course of their therapy [236]. Patients can present with thrombosis at any site (both venous and arterial), with a predilection for unusual sites. Up to 19% of patient may have a visceral vein thrombosis at the time of diagnosis [237]. Budd Chiari is rare, but has a strong association with PNH with up to 19% of Budd Chiari patients eventually diagnosed with PNH [238]. Thrombosis risk correlates with the size of the aberrant clone, but even patients with small clones <10% still have a significantly increased risk of thrombosis [236, 239]. The cause of the hypercoagulable state is not fully understood, but complement activated platelets are the likely driver, along with complement activated neutrophils [240–242]. Other likely causes include hemolysis driven events including toxicity from free hemoglobin, decreased nitric oxide, and release of procoagulant red cell microparticles, all of which may contribute to prothrombotic endothelial dysfunction [236].

A treatment for PNH is the complement inhibitor eculizumab, a humanized monoclonal antibody which targets complement protein C5, thereby preventing assembly of the terminal complement complex during complement activation. In studies, treatment with eculizumab has been shown to reduce hemolysis, as well as improve anemia, fatigue, and quality of life in patients with PNH [243]. Additionally, eculizumab has demonstrated efficacy in reducing thrombosis. In an aggregate review of rates of thromboembolism in three major clinical trials, the event rate with eculizumab treatment was 1.07 events/100 patient-years compared with 7.37 events/100 patient-years ($P < .001$) prior to eculizumab treatment (relative reduction, 85%; absolute reduction, 6.3 TE events/100 patient-years) [244].

Patients presenting with acute thrombosis should receive immediate anticoagulation, and this should be continued indefinitely. If not already on eculizumab, this agent should be started. Studies are underway to see if use of eculizumab therapy may allow cession of anticoagulation. In the past breakthrough thrombosis does occur even in patients on anticoagulation but as noted above this is rarely seen in the eculizumab era [239, 245].

Abnormal Proteins

Dysproteinemias

Although uncommon, defects in coagulation and platelet function may pose serious problems in the management of patients with dysproteinemias secondary to multiple myeloma, lymphoplasmacytic lymphoma, and rarely other B-cell disorders which can produce paraproteins [246, 247]. Paraproteinemia at high enough levels can cause pathologic interactions with coagulation factors, platelets and vascular endothelium which can lead to abnormal hemostasis test results, and rarely both bleeding diathesis and thrombosis [248].

Bleeding symptoms seem to be more frequent with IgM and IgA paraproteins, in dysproteinemias associated with κ rather than λ light chains, and in patients with total protein concentrations greater than 8 g/dL or serum viscosities greater than 5 [249, 250]. These correlations may reflect larger size or amount of paraproteins disrupting coagulation factor and platelet function. Patients with prolonged bleeding times and decreased platelet adhesiveness also are at a higher risk of hemorrhage than those with normal platelet function [250]. While rates of bleeding are lower with myeloma, at presentation up to 17% of patients with lymphoplasmacytic lymphoma may have bleeding complications [251, 252].

Multiple hemostatic abnormalities have been described in patients with dysproteinemias (Table 59.4). First, the physical structure of the fibrin clot may be abnormal, owing to increased serum globulins. Electron microscopy often shows an amorphous collection of proteins covering the fibrin strands, producing clots more gelatinous and susceptible to local fibrinolytic enzymes than normal [253]. Clot retraction is absent in some patients with a dysproteinemia because the abnormal protein prevents the attachment of platelet pseudopods to one another or to fibrin strands [253].

Table 59.4 Abnormalities of coagulation in myeloma and amyloid

Abnormally low clot retraction
Abnormally low and weak fibrin clot
Anti-glycoprotein IIb/IIIa antibodies leading to platelet dysfunction
Factor VIII inhibitor leading to low factor VIII levels
Factor X deficiency (amyloid)
Heparin-like anticoagulant
Impaired fibrin polymerization leading to poor thrombus formation
Inhibition of thrombin time indicative of impaired fibrin formation
Systemic fibrinolysis (amyloid)

Polymerization of fibrin is also impaired in some patients with circulating λ light chains, which is suggested by prolonged thrombin and reptilase times [254]. Myeloma proteins have also been shown to inhibit the thrombin time of normal plasma [254, 255]. More detailed studies have demonstrated that the antibody-binding portion of the myeloma protein, rather than the heavy chain, is involved in the inhibition of α-chain polymerization of fibrinogen [254]. The receptor for factor XIIIa (Also known as fibrin stabilizing factor) on fibrin strands can be blocked by an abnormal paraproteins leading to increased circulating level of factor XIII [256, 257]. Lastly, some patients with plasma cell leukemia have a potent heparin-like anticoagulant that produces very severe and often fatal bleeding (as discussed later in this chapter) [258].

Platelet abnormalities are less well defined in patients with myeloma and macroglobulinemia, although prolonged bleeding times and abnormal platelet adhesiveness have been described [250, 259]. The proposed mechanism is coating of platelets by immunoglobulins in a nonspecific fashion [252]. In one instance, a purified IgG_1-κ paraprotein was shown to bind to platelet glycoprotein IIIa inhibiting platelet aggregation and provoking a severe bleeding disorder [260].

An often overlooked and rare cause of bleeding is an acquired von Willebrand disease (VWD) which can occur with paraproteinemia [252, 261]. Over 450 cases of acquired VWD have been reported, likely being generated through a variety of different mechanisms, with a majority associated with paraproteinemia from a malignancy or monoclonal gammopathy of unknown significance (MGUS) [262]. Patients often present with laboratory findings similar to type 2A VWD including abnormal bleeding times, markedly reduced von Willebrand factor (VWF) activity, decreased VWF activity-antigen ratio, absence of large multimers, and normal plasma concentrations of VWF propeptide [263, 264]. VWF autoantibodies can rarely be detected with mixing studies, although laboratory analysis of these antibodies ifs difficult and it is unclear if this is the paraprotein itself or an associated secondary antibody [252, 265]. Acquired VWD is discussed more in detail later in this chapter.

Therapy for the hemostatic defects in the dysproteinemic syndromes include removal of the offending protein, either by reducing synthesis by treating the plasma cell dyscrasia or by intensive plasmapheresis. In several patients, return of normal hemostasis correlated with a substantial reduction in the paraprotein spike in the serum [250, 266].

Increasingly there is evidence that dysproteinemia are associated with an increased risk for thromboses as well. There are scattered case reports of paraproteins with lupus anticoagulant activity associated leading to deep venous thrombosis [267]. Inhibitors leading to acquired protein S and protein C deficiency have been reported [268, 269]. Acquired protein C resistance may be relatively common in multiple myeloma, and has been correlated to thrombotic risk [270]. Observational studies suggest that dysproteinemias irrespective of malignancy may be a common risk factor for thrombosis. Several studies have indicated that MGUS can be associated with a 2–3-fold risk of thrombosis, both arterial and venous [271, 272].

Amyloidosis

Patients with systemic amyloidosis, either primary or associated with myeloma show a marked increase in easy bruisability and other bleeding symptoms [273]. Abnormal coagulation testing is common in patients with amyloidosis with an elevated in thrombin time seen in 30–80% of cases, increased prothrombin time (PT) in 17–24%, and an increased partial thromboplastin time (aPTT) in 70% [274, 275]. Factor X deficiency was first reported to be associated with amyloidosis in 1962; subsequent studies have shown that the clotting factor is adsorbed onto amyloid proteins [247, 276, 277]. Splenectomy, plasmapheresis, and treatment with high-dose chemotherapy and auto stem cell transplant have all been reported to reduce the amyloid burden and to increase the levels of factor X [277–279]. Many of the bleeding symptoms from amyloidosis are also due to amyloid infiltration of blood vessels, rather than to coagulation or platelet defects [273]. Consistent with this idea is a study of 60 patients with factor X levels <50%, only line placement was associated with bleeding [280]. An observation study of 368 individuals with systemic AL amyloidosis found that 8.7% had factor X levels below 50% [277]. Another large case series at another institute reported a 14% incidence of low factor X with 5% of patients having levels below 20% [274].

Two other hemostatic defects have been reported to complicate primary amyloidosis. In one instance, circulating Bence-Jones protein formed complexes with plasma fibrinogen and interfered with the polymerization of fibrin monomer as evidenced by a prolonged thrombin time [281]. This, along with similar mechanisms as outlined in the section on dysproteinemia, may explain why abnormal thrombin and reptilase times are found in up to 30% of patients [274]. In another report, a circulating inhibitor to factor VIII was found to be an IgA-κ monoclonal protein similar in structure to the amyloid subunit protein [282].

Yet another cause of bleeding in patients with systemic amyloidosis is systemic fibrino(geno)lysis [283–286]. The euglobulin clot lysis time is shortened with striking decreases in α_2-PI, plasminogen, and circulating plasmin-antiplasmin complexes. Some patients have also been reported to have elevated plasma levels of tissue-type plasminogen activator, which can normalize with treatment of the amyloidosis [285, 287–289]. One small study found that more than 90% of bone marrow plasma cells from five different AL-amyloidosis patients were positive for a urokinase type-plasminogen activator by immunohistochemical staining [286]. The complete mechanisms responsible for the fibrinolytic state are not known is likely due to increased release of plasminogen activators or decreased plasminogen activator inhibitors from blood vessels infiltrated with amyloid, decreased α_2-PI because of its adsorption onto amyloid fibrils, or perhaps amyloid associated liver disease. The use of fibrinolytic inhibitors such as EACA or tranexamic acid have both corrected laboratory tests of fibrinolysis and reduced bleeding symptoms [289].

Immune Thrombocytopenic Purpura

Immune thrombocytopenic purpura (ITP) is not commonly associated with hematologic neoplasms, but has been reported in 2–4% chronic lymphocytic leukemia, and in approximately 2% of patients with Hodgkin's disease, but are less commonly seen as a complication of non-Hodgkin's lymphoma. ITP is known to predate the onset of Hodgkin's disease, to occur at the time of diagnosis, to indicate a recurrence (within the spleen), or to develop in disease-free individuals long after they have been considered cured [290–294].

The presentation of ITP in patients with lymphoproliferative diseases is usually not different than sporadic cases of ITP and is associated with decreased platelet survival and elevated levels of platelet associated IgG [294–297]. Why ITP occurs concurrently with some lymphoproliferative disorders is not well understood, although a disturbed immune system could possibly predispose patients both to the lymphoproliferative disorder and to thrombocytopenia [298, 299]. Rare cases of acquired Glanzmann thrombasthenia have been reported with Hodgkin's disease and other lymphoproliferative disorders [300, 301]. Acquired thrombasthenia should be considered when bleeding appears to be out of proportion to the decrease in platelet count.

Therapy for the ITP that complicates Hodgkin's disease or chronic lymphocytic leukemia does not differ from that for classic immune thrombocytopenia, and often overlaps with treatment of the lymphoproliferative disorder [302–305]. High-dose corticosteroids such as pulse dexamethasone 40 mg/day × 4 days, and intravenous immunoglobulins (IVIg) are generally given first [306]. For patients with presumed, but as yet undiagnosed lymphoma, steroids should be avoided if possible, until an adequate biopsy is obtained for diagnosis. If an adequate platelet count cannot be maintained and bleeding is present splenectomy can be considered. Rituximab therapy alone may be effective enough such that splenectomy can be avoided in certain patients, especially those whose comorbidities make splenectomy risky [306–308]. In the past if removal of the spleen failed to increase the platelet count, immunosuppressive therapy with vincristine, azathioprine, or pulse cyclophosphamide was tried [309, 310]. However, more recent clinical trials show a high rate of success with the thrombopoietin agonists romiplostim and eltrombopag and use of these agents should be considered as the next line of therapy [311, 312].

A search for recurrent Hodgkin's disease should be considered in patients who develop ITP after successful remission is obtained with chemotherapy. ITP does occur in the absence of lymphoma relapse, but caution must be used to ensure no active relapsed lymphoma is present before initiating steroids or chemotherapy which may alter the sensitivity of pathologic evaluation. ITP has even occurred in patients with Hodgkin's disease who have had splenectomy or who were receiving intensive immunosuppressive therapy [291, 298, 313–315].

Acquired von Willebrand Disease and Other Coagulation Inhibitors

Acquired von Willebrand disease (VWD) can complicate lymphomas, myeloproliferative syndromes, myeloma, and monoclonal gammopathies [252, 261, 263, 265, 316, 317]. The most common presentations include diffuse oozing from surgical sites, epistaxis, or gastrointestinal bleeding [318]. Patients with acquired VWD can present as type 1 (decreased protein) or type 2 (abnormal multimers) disease which can be)differentiated via crossed-immunoelectrophoresis [265, 319]. Levels of factor VIII, ristocetin cofactor activity, and von Willebrand antigen are all decreased [265]. Platelet levels of VWF are normal, suggesting depletion of circulating VWF from the plasma.

There are several mechanisms that are through to lead to acquired VWD. The first is the presence of circulating autoantibody against the VWF which can either produce increased VWF clearance [320], or interfere with platelet or collagen binding [265, 321, 322]. The second mechanism is increase absorption of the protein onto abnormal lymphoid cells or platelets in the setting of thrombocytosis [323–326]. For example, a case report demonstrated the aberrant expression of glycoprotein Ib by a marginal zone lymphoma results in acquired VWD [327]. Lastly, increased proteolysis of the von Willebrand proteins has been reported, especially in myeloproliferative disorders [167–169].

Patients with acquired VWD have variable responses to therapy. Therapeutic measures have included infusions of cryoprecipitate or concentrates containing vWF, DDAVP, plasma exchange, intravenous immunoglobulin, or splenectomy (when VWF was shown to bind to the abnormal lymphocytes) [167, 265, 324, 328, 329]. Desmopressin is effective at controlling bleeding in many patients with acquired VWD type 1 and 2, but often has subpar effect at control of bleeding in patients with associated myeloproliferative (21% success rate), or lymphoproliferative (44% success rate) disorders [330]. VWF levels should be checked with therapy. High-dose intravenous immune globulin (1 g/kg) is also effective in reversing acquired VWD by decreasing antibody levels, especially if associated with IgG monoclonal gammopathies, except for those with IgM paraproteins [331, 332]. For patients with significant bleeding, high doses of Humate-P are indicated with frequent monitoring of factor VIII levels [331]. A large series of patients with acquired VWD has suggested the use of recombinant factor VIIa to be effective when other measures had failed [333, 334]. Whenever possible, treatment of the underlying condition should also be undertaken. Cytoreduction, for instance, has been shown to cause remission of acquired VWD in patient with myeloproliferative disorders [335].

Rarely in patients with lymphoproliferative disease, acquired factor VIII inhibitors have been reported [282, 336, 337]. An inhibitor to prothrombin that accelerated clearance of the protein has also been reported [338]. An antibody directed against thrombin has also been reported in a myeloma patient with plasmapheresis being effective in reducing bleeding [339].

Finally, circulating coagulation inhibitors with heparin-like properties have been described in patients with multiple myeloma and other neoplasms [340–343]. These patients present with very severe bleeding along with elevated aPTT's, and thrombin times, but a normal reptilase time. The disease is caused by circulating proteoglycan similar to heparin. This proteoglycan has antithrombin enhancing activity, and can be blocked by protamine sulfate, platelet factor 4, or toluidine blue in vitro [340, 342, 343]. These heparin-like anticoagulants regularly cause severe and unrelenting bleeding which is often fatal. Therapy is difficult but has included intensive chemotherapy to reduce the malignant cell burden, plasmapheresis to remove the anticoagulant, and intravenous protamine sulfate [343].

Therapy-Induced Bleeding and Thrombosis

L-Asparaginase

L-Asparaginase is an enzyme from bacteria that catalyzes the hydrolysis of asparagine, an amino acid required by leukemic, but not normal cells. When used as a chemotherapeutic agent, it has a number of side effects, including hypersensitivity reactions, pancreatitis, liver insufficiency with decreased protein synthesis, neuropathy, and somnolence [344].

l-Asparaginase also lowers circulating hemostatic, anticoagulant, and fibrinolytic factors, with substantial decreases in plasma fibrinogen, factor IX, factor XI, antithrombin III, protein C, protein S, and plasminogen [345–354]. These effects are most likely due to diminished protein synthesis by the liver; there is no evidence for clotting factor consumption or the production of abnormal molecules [355, 356]. This reduction in protein synthesis appears to be in proportion to the effectiveness of l-asparaginase eliminating asparagine from the plasma [357]. As soon as therapy is discontinued, the clotting factors return rapidly to normal. Fortunately, l-asparaginase does not interfere with platelet production, and platelet counts return to normal even during continued administration for remission induction in patients with ALL [358, 359].

The concentrations of the anticoagulant proteins antithrombin III, protein C, and protein S can fall to 30% of normal in some patients receiving l-asparaginase, levels low enough to predispose to thrombosis [348, 360]. Levels of antithrombin III biologic activity have generally paralleled reductions in antithrombin III antigen, although exceptions may occur [360]. Alterations in the multimeric composition of VWF and variable defects in platelet function have also been reported [354, 361].

At first, the coagulopathy induced by l-asparaginase was attributed to DIC. Low levels of fibrinogen, increased FDP, a positive protamine sulfate test for fibrin monomer, and low levels of factor V and factor VIII have all been reported [351]. However, more current studies have shown normal survival of labeled fibrinogen and no evidence of increased thrombin generation [356, 362]. Some of the early reports of DIC may have represented patients who were predisposed to intravascular clotting because of their underlying disease, which then became overt due to l-asparaginase-induced decreases in circulating antithrombin III or other anticoagulant proteins. Although marked reductions in fibrinogen, factor XI, and factor IX due to l-asparaginase have been observed frequently, excessive bleeding is rare [345, 355]. If bleeding does occur, cryoprecipitate or fresh frozen plasma rapidly corrects the hemostatic defects, however fresh frozen plasma should be avoided if possible as this contains asparagine and may inhibit the antileukemic effects of the therapy.

The overall rate of thrombosis in children is 5% but may be as high as 36% if asymptomatic thrombosis are included and can range from 5 to 18% in adult patients with the suggestion the rate may be lower with PEG-asparaginase. The rate of potentially devastating CNS thrombosis is approximately 1–2% of patients with childhood ALL and up to 4% of adults [344, 352, 356, 363–367]. Small cerebral blood vessels can be occluded by thrombus, producing bland or

hemorrhagic infarction. Dural sinus vein thromboses are also common. Thrombosis usually occurs 2–3 weeks after the start of a course of therapy. Most patients recover, although several deaths have been reported and others are left with debilitating neurologic defects. Peripheral deep venous thrombosis seems to be less common [364]. Then presence of a central catheter increases the risk of thrombosis [368].

The pathogenesis of the thrombotic complications of l-asparaginase therapy remains obscure, although decreased levels of antithrombin III, protein C, protein S, and plasminogen could predispose to thrombosis because of inadequate inhibition of circulating activated clotting factors or deficient fibrinolytic activity. The low levels of circulating fibrinogen do not seem to be protective. A patient has been reported for instance who developed a major CNS thrombosis even though the plasma fibrinogen had fallen to 20 mg/dL [364]. The presence of an inherited hypercoagulable state such as factor V Leiden or the prothrombin gene mutation raises the risk of thrombosis with l-asparaginase therapy [368–370].

Optimal therapy of the thromboembolic complications of the l-asparaginase depends on whether intracerebral bleeding or thrombosis is identified on computerized tomography (CT) or magnetic resonance imaging (MRI) scans. If primary intracranial hemorrhage is present, heparin should not be used and coagulation defects corrected. If it is venous thrombosis then systemic anticoagulation with heparin is indicated [371]. Prior to heparin, measuring of fibrinogen should be done—if low (<150 mg/dL) then it should be replaced before anticoagulation. Some authors advocate replacement with antithrombin III and cryoprecipitate (for ATIII level < 60 and fibrinogen <150 mg/dL, respectively) [39]. Fresh frozen plasma should be avoided if possible, as previously stated.

There remains no consensus on prevention of thrombosis given varying results of clinical trials. Most would recommend check aPTT, INR, antithrombin, and fibrinogen as the start of asparaginase therapy and then perhaps every other day for a total of 7 days after each dose. The cutoff for antithrombin replacement remains undefined with levels of 60 or 70% recommended by different centers [272, 372]. In pediatric studies LMWH prophylaxis may be useful for patients with high risk for thrombosis, high WBC counts, or central venous catheters [373]. However, with the lack of clinic trial data no firm recommendations can be made.

Dasatinib and Imatinib

Recent reports of bleeding have been noted in patients with CML undergoing therapy with both first and second generation tyrosine kinase inhibitors [374]. In one retrospective study, multivariate analysis identified thrombocytopenia and advanced phase CML as risk factors for bleeding in patients during dasatinib therapy. Another study from the same group demonstrated that both dasatinib and imatinib induced rapid and marked prolongation of closure time to collagen/epinephrine in normal whole blood on the PFA-100 system, thus theoretically affecting coagulation via abnormalities in platelet function [375].

Ibrutinib

Within the last few years the targeted Bruton's tyrosine kinase (BTK) inhibitor ibrutinib has been trialed in several hematologic malignancies with good effect, however, high rates of bleeding events have been reported with a up to a 44% incidence of low grade, petechial or ecchymotic bleeding [376–379]. The drug appears to inhibit BTK activity in platelets as well as lymphoid cells, which functions in the signaling pathway of the platelet collagen receptor glycoprotein VI (GPVI) [380]. This has been confirmed by the observation that individuals taking the drug have deceased platelet aggregation to collagen, but not to ADP [380, 381]. The drug has also been shown to affect the adhesion of platelets with VWF [382]. Initial trials allowed patients to use concurrent vitamin K antagonists; however these patients were later removed from trials due to an increased rate of bleeding [383]. Concomitant use of vitamin K antagonists should be avoided.

Antibiotic-Induced Coagulopathies

Antibiotics have been recognized as a cause of bleeding in patients treated for infectious complications of hematologic neoplasia. Two categories of hemostatic defects have been described. First, vitamin K-dependent clotting factor synthesis can be impaired, leading to prolonged prothrombin or partial thromboplastin times [384, 385]. Second, platelet function is inhibited by certain β-lactam antibiotics such as high-dose penicillin, carbenicillin, and ticarcillin [385–390].

The mechanisms underlying the reduction of the vitamin K-dependent clotting factors are not clear, although the antibiotics can destroy the bowel bacteria that produce vitamin K [385, 391]. Alternatively, decreased levels of hepatic clotting factor synthesis or inhibition of the posttranslational gamma-carboxylation reaction required for the biologic activity of the vitamin K-dependent clotting factors could occur. The N-methylthiotetrazole side chain found in some of the cephalosporins can inhibit the action of vitamin K [385, 391–393]. Bleeding seems to be more frequent in severely debilitated patients with poor nutrition or liver disease, or those receiving prolonged intravenous feedings. The hemostatic defect can appear within 1 week of starting antibiotic therapy. Fortunately, the disorder may be quickly reversed or

prevented by therapeutic doses of oral or parenteral vitamin K or, if severe, and the patient is bleeding, fresh frozen plasma [385, 393, 394]. Prophylactic vitamin K has been suggested for leukemic patients who are likely to require long-term antibiotics; however, this intervention has been studied prospectively in children and was not found to be protective against development of a coagulopathy [395–398].

The antibiotic-induced defect in platelet function has been described with some, but not all β-lactam antibiotics and the incidence appears to be less with newer β-lactams. Susceptible patients can have markedly prolonged bleeding times, and experience marked bleeding after surgery or other invasive procedures [385, 388, 389, 399]. The incidence of antibiotic-induced platelet dysfunction is not known, although it is higher in debilitated, uremic, or malnourished patients. When healthy subjects are treated for up to 2 weeks, bleeding times are normal or only slightly prolonged [387, 388, 393, 400, 401].

Finally, certain antibiotics can induce immune thrombocytopenia [402]. While numerous drugs can produce thrombocytopenia, Common antibiotics such as vancomycin, piperacillin-tazobactam, and ciprofloxacin have all been implicated in thrombocytopenia and refractoriness to platelet transfusion [403–406]. The mechanism mimics HLA alloimmunization [404, 407]. In two instances antibodies were discovered that were vancomycin dependent and bound to glycoprotein IIb and/or IIIa [407]. Other investigators have isolated IgG and IgM vancomycin-dependent, platelet-reactive antibodies in vancomycin exposed patients that develop thrombocytopenia, which are not present in vancomycin exposed patients with normal platelet counts [408]. Also, antifungals such as amphotericin B can produce both thrombocytopenia and decreased recovery of transfused platelets, as well as rarely fluconazole [409, 410].

Chemotherapeutic Agents and Thrombosis

Chemotherapy is an independent risk factor for thrombosis [411]. Adjuvant chemotherapy for breast cancer for instance has been associated with an increased risk of both arterial and venous thromboembolism (5–7% of patients) [412–414]. The thrombogenic stimulus is not fully understood, but likely is resultant from a combination of damage to the vascular endothelium, increased levels of procoagulant molecules, reduction in endogenous anticoagulants including protein C and/or protein S, cytokine release from tumor cell death, induced platelet activation, and increased expression of macrophage tissue factor [411, 415–417]. Although thrombosis prophylaxis is not currently recommended for outpatients receiving chemotherapy [418], very low levels of warfarin (INR 1.5) or prophylactic LMWH can prevent the thrombotic events [419].

The multiple myeloma disease modifying agents thalidomide and lenalidomide are both associated with substantial rates of thrombosis that can be as high as 36–75% [246, 247]. The incidence is higher with the use of dexamethasone and with chemotherapy, especially doxorubicin [420]. These agents may have a direct toxic effect on the vascular endothelium promoting a prothrombotic state. Aspirin appears useful for thrombosis prevention in low risk patients while those who have had previous thrombosis, receiving dexamethasone or chemotherapy, immobilization, inherited thrombophilia, or have central lines may benefit from warfarin or LMWH prophylaxis [421]. LMWH prophylaxis has been compared to aspirin prophylaxis and warfarin prophylaxis in a prospective clinical trial [422]. The authors concluded that LMWH lead to the lowest risk of VTE, although this was not statistically different. Current guidelines recommend tailoring prophylaxis strategies to the patient's thrombotic risk factors [421].

Therapy-Related Thrombotic Microangiopathy

Thrombotic microangiopathy (TM) syndromes can complicate a variety of therapies for hematologic malignancies and their treatments [423, 424] (Table 59.5). TM can be associated with medications such as the calcineurin inhibitors cyclosporine, tacrolimus, as well as gemcitabine, and clopidogrel. Calcineurin inhibitor associated TM can occur within days after the agent is started manifesting as a falling platelet count, falling hematocrit, and rising serum LDH level [423, 425]. Some cases have been fatal but often the TM resolves with decreasing the dose of the calcineurin inhibitor or changing to another agent. Calcineurin inhibitors likely have directly toxic effects leading to endothelial dysfunction and increased platelet aggregation, possibly through the inhibition of prostacyclin [426].

In the past TM was most commonly seen with the antineoplastic agent mitomycin C, with a frequency of 10% when a dose of more than 60 mg was used [427]. Now, the most common

Table 59.5 Diagnostic criteria for stem cell transplantation related TTP/HUS

Bone marrow transplant clinical trial network criteria [481]
1. RBC fragmentation and ≥2 schistocytes per high powered field
2. Concurrent increase in LDH from institution baseline
3. Concurrent renal and/or neurological dysfunction with no other explanation
4. Negative Coombs test
International Working Group [482]
Increased percentage (>4%) of schistocytes in the blood
1. New, prolonged or progressive thrombocytopenia (< 50,000/uL or >50% decrease from previous counts)
2. Sudden and persistent increase in LDH
3. Decreased hemoglobin or increase transfusions requirements
4. Decreased in serum haptoglobin

antineoplastic drug causing TM is likely gemcitabine [428, 429]. The appearance of the TM syndrome associated with gemcitabine can be delayed, and the condition often is fatal. Severe hypertension often precedes the clinical appearance of the TM [430]. The use of plasma exchange is controversial, particularly as advanced malignancy itself can be associated with a TM-like syndrome that is typically poorly responsive to plasma exchange. There is increasing data that eculizumab may be effective [431]. TM has also been associated with cisplatin and VEGF inhibitors [432, 433].

Stem Cell Transplantation

Hepatic veno-occlusive disease (VOD or sinusoidal obstruction syndromes) is a relatively common complication of stem cell transplantation and is seen in 1–50% of patients, but the frequency seems to vary widely from center to center and the conditioning protocol used [434–436]. The clinical syndrome includes weight gain, hepatic tenderness, and jaundice soon after transplantation which can progress to liver failure and the hepatorenal syndrome. In older data hepatic VOD was common with rates up to 54% with a mortality of 39% but with use of less toxic preparatory regimens rates in the current era average 11% [437, 438].

Early thrombosis of the hepatic venules leading to obstruction and eventual fibrosis is the most commonly accepted mechanism for VOD [434]. Preexisting liver dysfunction, especially hepatitis C, prior therapy with gemtuzumab ozogamicin, the use of vancomycin in the pre-transplant period, and advanced age are some of the important risk factors for development of the disorder [434, 439–442]. Conditioning regimens that include Busulfan also increases the incidence [434, 437]. The risk is higher in patients undergoing allogeneic rather than autologous transplantation, unrelated donor transplants, and HLA mismatched transplants [437, 443].

Multiple coagulation defects have been demonstrated, but low levels of protein C prior to transplant were a strong and reproducible predictor of VOD [444–447]. For example, all patients with a baseline protein C value of less than 66% of normal developed the syndrome [446]. At present, it is unclear whether the decreased level of protein C is simply a surrogate marker for underlying liver disease, or constitutes a specific pathogenetic mechanism. Elevated levels of plasminogen activator inhibitor-1 have been suggested as a noninvasive test for VOD [448, 449]. Factor V Leiden mutation and the prothrombin 20210 mutation have also been reported as potential risk factor for the development of VOD [186]. Prothrombotic cytokines such as TNF and IL-6 have been shown to be elevated in patients with VOD, and markers of activation of hemostasis such as F1.2 and TAT are also increased [450].

Antithrombotic therapy has been employed in an attempt to prevent against VOD in high-risk patients. A randomized trial demonstrated that heparin at a dose of 100 U/kg/day beginning 8 days prior to transplant and continuing for 30 days thereafter decreased the rate of VOD from 13 to 2.5%, and in patients receiving allogeneic transplants from 18 to 0% [451]. Thrombolytic therapy with urokinase or tissue plasminogen activator (t-PA) has been trialed for treatment of patients with established VOD [452]. A small pilot study of 7 patients with severe VOD treated with 10 mg/day × 2 days of t-PA followed by heparin showed a response in 5/7 patients but a larger follow-up study demonstrated a high risk of bleeding, especially in patients who already had developed multi-organ system failure [453]. Current guidelines recommend against the use of t-PA due to the associated risk of hemorrhage [454].

Early reports indicating the use of defibrotide—an oligonucleotide with multiple anti-inflammatory actions—is effective in the treatment of VOD [455, 456]. This was recently confirmed by a large phase III trial of defibrotide compared against historical controls in which 102 patients were given defibrotide 25 mg/kg per day [457]. Complete resolution occurred in 25.5% of patients receiving defibrotide and 12.5% of control patients, with significantly higher day 100 survivals in the group receiving defibrotide. Rates of hemorrhage were not dissimilar between the two groups. Previous trials of defibrotide have suggested that predictors of survival included younger age, autologous SCT, and abnormal portal flow, whereas busulfan-based conditioning and encephalopathy predicted worse outcome. Decreases in mean creatinine and plasminogen activator inhibitor1 (PAI-1) levels during defibrotide therapy predicted better survival [438, 458, 459].

Thrombotic microangiopathies (TM) can complicate both autologous and allogeneic hematopoietic stem cell transplants [460–463]. The reported incidence varies widely in the literature from 0 to 74% which is likely a reflection of poor understanding of the disease and a lack of standard definitions [464–466]. Rates are significantly higher with allogeneic transplant. Risk factors include high-dose chemotherapy, radiation, unrelated donor, HLA mismatch, exposure to calcineurin inhibitors (CNIs), graft-versus-host disease (GVHD), and systemic infections [467, 468]. The etiology of post transplantation TM is felt to be different than that of "classical" TM, more likely being secondary to direct endothelial damage and subsequent release of cytokines, as ADAMTS13 levels are generally unchanged [460]. Complement dysfunction has been suggested as well [469, 470]. Mortality rates are quite high, with reported rates up to 100% [471].

Patients may present with a fulminant multi-organ failure, which occurs early after transplantation (e.g., within 20–60 days) which has been associated with severe cytomegalovirus (CMV) infection and is often fatal [472]. TM can be directly caused by cyclosporine/tacrolimus as outlined previously in this chapter. TM that is associated with the

conditioning regimen used in the transplantation protocol can occur 6 months or more after total body irradiation, and is associated with primary renal involvement.

Given the diagnosis of any TM is a clinical one and transplant patients are prone to have many complications that can mimic TM, it is easy to see why there is great center to center variation in incidence. Two groups have proposed diagnostic criteria that share the common features of evidence of a microangiopathic hemolytic anemia and elevated LDH. However, applying these criteria to an individual patient still require clinical judgment (Table 59.5).

The best management of hematopoietic stem cell transplantation-related TM is uncertain. For cyclosporine/tacrolimus associated TM the initial therapy is stopping the medications. In patients with mild TM and high serum levels of these agents one can lower the dose to see if the symptoms abate. For conditioning TM the course is progressive with no specific therapy available. For systemic CMV associated TM aggressive treatment of the CMV is the cornerstone of therapy. Treatment modalities for fulminant TM with multi-organ failure remain unsatisfactory. First steps include treatment of any process that may be aggregating the TM such as GVHD, infections, etc. Unlike in classic TTP, the role of plasma exchange remains controversial. Most series report very poor response rates and outcomes with high rates of complications. It is common the patient may respond for a few days but then relapse. A practical approach would be to use one plasma volume/day exchange daily in patients with TM until it is clear they are not responding to therapy [473–475]. Small series have evaluated the role of agents such as eculizumab [476], rituximab [477], vincristine [478], defibrotide [479], and pravastatin [480] with varying results. Unfortunately there remains no satisfactory treatment approach.

Summary

Hematological neoplasms can have protean effects on hemostasis. Not only can these be due to the tumor itself but can also be a complication of therapy. Knowledge of what complications to expect with both the tumor type and treatment can help prevent bleeding and thrombotic complications.

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60 Psychological Aspects of Hematological Neoplasms

Tomer Levin T. Levin and Judith Cukor

Introduction

In an era of evidence-based medicine, it is important to choose psycho-oncology treatments that are as efficacious as possible. Psychosocial cancer care that is ineffective may be worse than no care at all. The ultimate aim of psychosocial care is to facilitate better medical outcomes.

Approximately, one-third of oncology patients will have significant psychological distress. Oncologists are often first responders for treating psychological problems; hence, there is a great need to equip them with solid psycho-oncologic skills. Clinicians should have demonstrable basic competence in managing disorders such as anxiety, depression, panic, alcohol withdrawal, delirium, and aptitude in handling physician–patient–family communication.

Furthermore, all oncology departments should have psycho-oncology consultation and referral resources for helping their patients, families, and clinical staff. This means investing in psycho-oncology programs and collaborative networks with other psychosocial care providers such as social workers, chaplains, integrative medicine, patient representatives, survivorship groups, and online support networks. A team approach is vital.

This chapter focuses on clinically substantiated interventions that are practically useful to the clinician. It will discuss anxiety, depression, delirium, psychosocial challenges of transplant, cognitive sequelae of treatments (chemo brain), doctor–patient communication, and physician burnout. It will also review a new population-based approach called Integrative and Collaborative Care.

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Anxiety

What Is Anxiety?

Anxiety is an unpleasant psychobiological response, activated in response to a perceived threat. It allows an individual to mobilize resources to minimize exposure to a danger, and in so doing, triggers the primal fight, flight, or freeze reaction. The anxiety phenotype has cognitive, emotional, behavioral, and physiological elements (Table 60.1) [1].

Anxiety, like pain and fever, is not necessarily pathological. An optimal dose of anxiety can motivate the individual to take action to minimize a threat. For example, worry is positively associated with increased breast cancer screening via both self-examination and mammography ($r = 0.12$) [2].

Anxiety becomes pathological when it is activated disproportionately to a threat, independently of an actual danger or when it becomes chronic. Such maladaptive anxiety has a

Table 60.1 Components of the anxiety phenotype

Cognitive	Evaluation of the danger versus one's coping resources. Focus is on the perceived threat while extraneous environmental details are blocked out (tunnel vision). For example, a patient may focus on the risk of stem cell transplant to the exclusion of marital or financial issues
Emotional	Feeling nervous, edgy, scared, alarmed, terrified, worried, etc.
Behavioral	This refers to behaviors associated with anxiety: avoidance (e.g., nonadherence), flight (e.g., signing out against medical advice), fight (e.g., angry or litigious), immobility, unable to speak (e.g., a passive or overtly shy patient)
Physiological	Cardiovascular (palpitations, hypertension, faintness/fainting), respiratory (tachypnea, chest pain, lump in throat), neuromuscular (startle reflex, twitching muscles, tremor, unsteady, restless), gastrointestinal (discomfort, anorexia, nausea, diarrhea, vomiting), urinary (urgency, frequency), dermatological (flushed, pale, sweaty, itchy, hot, shivers, blushing)

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noxious intensity that impairs a person's functioning and also wears out their family and friends because, unlike effective problem-solving, it serves no end point.

Consider an anxious chronic lymphocytic leukemia patient receiving watchful waiting treatment. Significant effort may be expended on fruitless worry, hypervigilance, self-palpation of lymph nodes, and amplification of nonspecific physical symptoms. All of these do not improve prognosis beyond that of a rational surveillance program. In this chapter, the term anxiety refers to the maladaptive variety rather than normal anxiety.

Whether anxiety is adaptive or pathological is moderated by a person's sense of self-efficacy or confidence in dealing with potential threats as well as the resources available to them. Loss of work or family roles, negative medical experiences such as misdiagnosis, or physical weakness leading to a self-image of a weak or vulnerable person, can all erode confidence and increase anxiety.

Prevalence of Anxiety

The prevalence of cancer anxiety is estimated to be in the 10–30% range, but this range should be assessed within the context of the ubiquity of anxiety in the general population (2–6%) and primary care (8–12%) [3, 4].

Risk Factors for Anxiety

Specific demographic factors such as younger age, female sex, separated, divorced, widowed, and lower socioeconomic status are associated with greater anxiety levels. Conversely, older, married, more experienced patients with social support and financial and educational resources can better manage anxiety [5, 6]. Nearing death or having a worse prognosis is probably not associated with increased anxiety [7, 8]. Genetic factors are also increasingly recognized as contributing to the risk for anxiety disorders; however, genes are not always the answer. For example, a spider phobia may have greater valence to a child as compared to an adult but, with appropriate nurturing, an anxious child may grow into a confident young adult. In other words, experience and maturation can override a biological propensity towards anxiety.

Finally, childhood sexual abuse is associated with an increased risk for generalized anxiety disorder and panic disorder (also major depression, substance abuse, and bulimia nervosa). This risk is doubled when the victim is subjected to sexual intercourse [9], highlighting the importance of considering sexual abuse when interviewing anxious cancer patients. Furthermore, because trust in parental figures has been betrayed at an early age, sexually abused oncology patients may have difficulty trusting their physicians and be more difficult to reassure.

Screening and Measuring Anxiety

Asking, "Are you worried?" or using the Distress Thermometer, where patients are asked to rate their distress from 0 to 10 [10], are simple ways of assessing anxiety. The Generalized Anxiety Disorder-7 scale (GAD-7), used widely in medical cohorts, matches DSM-IV criteria for generalized anxiety disorder (Table 60.2) [11]. It can be used to screen for anxiety where a

Table 60.2 Generalized anxiety disorder-7

Over the *last 2 weeks*, how often have you been bothered by the following problems?				
	Not at all	Several days	More than half of the days	Nearly every day
1. Feeling nervous, anxious, or on edge	0	1	2	3
2. Not being able to stop or control worrying	0	1	2	3
3. Worrying too much about different things	0	1	2	3
4. Trouble relaxing	0	1	2	3
5. Being so restless that it is hard to sit still	0	1	2	3
6. Becoming easily annoyed or irritable	0	1	2	3
7. Feeling afraid as if something awful might happen	0	1	2	3
	Total Score________ =	Add columns_________ +	______________ +	_______________

If you checked off *any* problems, how *difficult* have these problems made it for you to do your work, take care of things at home, or get along with other people?			
Not difficult at all	Somewhat difficult	Very difficult	Extremely difficult
❍	❍	❍	❍

Interpretation of scoring: 0–4 minimal anxiety (below threshold of significance), 5–9 low anxiety, 10–14 moderate anxiety, 15–21 severe anxiety. Developed by Drs. Robert L. Spitzer, Janet B.W. Williams, Kurt K. Kroenke and colleagues, with an educational grant from Pfizer Inc. No permission required to reproduce, translate, display, or distribute

cutoff of ≥5 represents significant anxiety. Alternatively, it can be used to measure anxiety severity, e.g., before and after starting a benzodiazepine.

Specific anxiety syndromes are discussed next.

Generalized Anxiety Disorder

When diagnosing generalized anxiety disorder (GAD) in psycho-oncology settings, the usual 6-month duration of symptoms is impractical because it would lead to underdiagnosis. A sensible clinical modification is a 2-week symptom duration [11]. Nevertheless, most GAD patients will have suffered from generalized anxiety, on and off, for many years previously; average duration of symptoms before diagnosis is 5–10 years. These patients tend to be greater users of primary and specialist medical care, have greater social impairment, demonstrate less work productivity and more absenteeism [3], and frequently display intercurrent depression.

How is GAD colored by the oncology setting? These patients may worry excessively about prognosis, uncertainty of recurrence, treatments, role changes, loss of income and status, transportation, and dependency. "Markeritis" describes overly worrying about elevated cancer markers. The "tyranny of positive thinking" is where patients are told that they "must" think positively, implying that negative thinking will worsen the prognosis. It is, however, impossible to be perpetually optimistic, even in the best of times, but especially when seriously ill. Thoughts about mortality are normal and understandable. Telling a cancer patient "not to think negative" is similar to saying, "don't think of a purple elephant"—quite impossible, and this increases anxiety.

Adjustment Disorder with Anxiety

The cardinal features of this category are anxiety symptoms within 3 months of a stressor that should resolve within 6 months of termination of the stressful trigger. The presumption is that, without the stressor, the patient would not have anxiety or worry symptoms.

The advantages of this classification are that it is not pejorative and semantically empathic with an expectation of recovery. Disadvantages are that it is hard to predict who will recover versus who will progress to chronic generalized anxiety. Even when cured, cancer remains a long-term stressor with multilevel effects on patient, family, employment, and finances, suggesting that it is overly simplistic to view the stressor as an on–off button. This categorization also invites the argument that anxiety is "normal" in a cancer setting, which may in turn lead to underrecognition and undertreatment, e.g., not initiating pharmacotherapy or psychotherapy.

Panic Disorder with or Without Agoraphobia

Panic attacks are discrete episodes of intense apprehension, fear, terror, or a sense of impending doom that peak within 10 min. They are associated with physical symptoms such as chest pain, shortness of breath, choking or smothering sensations, and a fear of going crazy or losing control. Agoraphobia, fear of being trapped in a crowded place where escape might be difficult or embarrassing, often is a consequence of panic attacks. When panic attacks become recurrent, with persistent worry about additional attacks or concern about its implications (going crazy, heart attack) or significant "avoidance" because of the attacks, panic disorder can be diagnosed. An isolated panic attack may not have great clinical significance in some patients but in others it can be a cardinal event setting off a cascade or fear and avoidance.

Panic in cancer patients may occur de novo or reflect an exacerbation of a preexisting panic. It is often underdiagnosed; one cancer center reported a fifth of referrals to its psycho-oncology service with panic symptoms [12]. Cognitively, panic is conceptualized as the misinterpretation of bodily symptoms, for example, a stem cell transplant patient who misinterprets pain and discomfort catastrophically. Occasionally, panic disorder presents as a patient who wants to leave hospital "against medical advice" or who suddenly refuses chemotherapy.

Social Anxiety Disorder

This is characterized by persistent fear that social scrutiny will be humiliating or embarrassing, resulting in severe anxiety or reactions such as avoidance, withdrawal, or paralysis. As this disorder is largely a chronic condition that starts in teenage years, these patients are often underachievers in life. The cancer journeys of such painfully shy and inhibited patients are uncomfortable. They have difficulty negotiating the medical bureaucracy and speaking up because their biggest fear is being the center of attention. Scars, radiation burns, disfiguring surgery, intrathecal catheters, or anything that makes the patient stand out in a crowd can exacerbate social anxiety. For these shy patients, obtaining a second opinion is commonly an anxiety wrought experience, which is often avoided, to the patient's ultimate detriment.

Specific Phobia

Seen in 3.5% of the general population, blood–injection–injury phobia may result in fainting during procedures and avoidance of injections, blood tests, or dental care. As self-injection becomes increasingly common, blood–injection–injury phobia should be considered in patients who refuse or

have difficulty self-injecting. Claustrophobia is very common in the setting of imaging (e.g., closed MRI scans) and radiation treatments.

Anticipatory Anxiety and Nausea

Anticipatory anxiety and nausea are classically conditioned responses (Pavlovian conditioning) to nausea induced by oncology treatments. For example, when patients, feeling nauseas, because they have just received chemotherapy, are offered a bite to eat, food can inadvertently be paired with the noxious stimulus of nausea. Anxiety and nausea are then generalized beyond the cancer treatment so that all food cues result in anxiety and nausea, resulting in food avoidance. Preexisting anxiety, younger age, vulnerability to motion sickness, prior emetic chemotherapy, and abnormal taste sensations, all predispose to anticipatory nausea and anxiety [13].

Anxiety Due to a General Medical Condition

No oncologist wants to misattribute anxiety as psychological when it actually is due to an underlying medical illness [14]. Medical causes of anxiety are listed in Table 60.3. Treatment is primarily that of the underlying medical illness, although this may not necessarily eliminate the anxiety.

Shortness of breath is a common pathway that increases anxiety. It is rare to encounter a patient with shortness of breath from a pulmonary condition, arrhythmia, sepsis, or blood loss who does not feel anxious.

Table 60.3 Medical conditions and medications associated with anxiety

Metabolic	Hyperkalemia, porphyria, hypo- and hypercalcemia, hyperthermia, hypoglycemia, hyponatremia, vitamin deficiencies, hypovolemia, sepsis
Neurological conditions	Pain, raised intracranial pressure, central nervous system neoplasms, postconcussion syndrome, seizure disorder, vertigo
Endocrine	Adrenal abnormalities, hyper/hypothyroidism, parathyroid abnormalities, pituitary abnormalities, pheochromocytoma, carcinoid syndrome
Cardiovascular	Arrhythmia, congestive heart failure, coronary artery disease, anemia, valvular disease, cardiomyopathy
Pulmonary	Hypoxia, pulmonary embolism, asthma, chronic obstructive pulmonary disease, pneumothorax, pulmonary edema
Medications/toxic conditions	Corticosteroids, bronchodilators, antipsychotics, thyroid preparations, theophylline, sympathomimetic agents, levodopa, serotonergic agents, psychostimulants, antibiotics (cephalosporins, acyclovir, isoniazid), interferon, caffeine, cocaine, marijuana, withdrawal states (alcohol, opioid analgesics, benzodiazepines, caffeine)

Pain is a common cause of anxiety in cancer patients. In one study of hospitalized cancer patients, the prevalence of pain was 96% for patients with anxiety as opposed to 80% for patients without anxiety [15]. Patients in severe pain appear diaphoretic, restless, and nervous. Anxiety assessment can only be completed after adequate pain relief. Anxiety often resolves after the pain is treated, reflecting the often heard truism that acute pain causes anxiety and chronic pain results in depression.

Substance-Induced Anxiety Disorder

Substance-induced anxiety is the direct effect of a drug, medication, or toxin. Bronchodilators raise the pulse rate, compounding the anxiety caused by air hunger. Anti-inflammatory cytokines such as interferons can cause anxiety and panic, perhaps via serotonergic and dopaminergic pathways, although they may be better tolerated than previously thought [16, 17]. Corticosteroids commonly cause anxiety, emotional lability, insomnia, agitation, and restlessness. Thyroxine, psychostimulants, sympathomimetic agents, serotonergic agents, anticholinergics, immunosuppressants, antihistamines, and certain antibiotics may all produce symptoms of anxiety. Akathisia (motor restlessness), when an extrapyramidal side effect of the antiemetics prochlorperazine and metoclopramide, is often misdiagnosed as anxiety. Substance withdrawal (alcohol, benzodiazepines, barbiturates, opioids, nicotine), frequently unrecognized, may present with sudden, intense anxiety and agitation.

Depression

What Is Depression?

A depressed person feels down, sad, hopeless, and loses interest in life, hobbies, and things that previously were a source of pleasure (anhedonia). Their facial expressions (affect) are sad and their overall demeanor and posture reflect their morose mood. In severe depression, nothing can brighten the affect, not even a little humor or encouragement. Psychiatrists describe this as nonreactive affect. Irritability, decreased concentration (e.g., "I can't read anything beyond the newspaper headlines"), middle insomnia (falling asleep but waking up after a few hours), loss of appetite or excessive eating, psychomotor slowing, and guilt (e.g., "If only I had …") are other symptoms.

Prevalence and Impact of Depression

The prevalence of depression in oncology ranges from 10 to 25%. It is frequently comorbid with anxiety. One study found that two-thirds of depressed oncology patients also

met anxiety criteria [5]. The treatment implications of this are that depression and anxiety should be targeted simultaneously, e.g., by starting an antidepressant together with a benzodiazepine or behavioral therapy.

Depression is not more severe in early compared to advanced disease (e.g., watch and wait vs. late stage CLL), nor in milder versus more demanding treatments (e.g., autologous stem cell transplant compared to allogeneic). Depression does not become more severe as death approaches [7], a fact that can be used to reassure patients and families who are concerned that depression is ubiquitous with the dying phase of an oncological illness.

Depression will worsen the quality of life and is associated with longer admissions and lesser adherence but, according to current research, does not trigger cancer or increase vulnerability to relapse [18].

Risk Factors for Depression

Of the malleable risk factors, substance abuse is common and of most urgent concern. In addition, less social support has been associated with depression and an overall increase in mortality of cancer patients [19]. It is hard to battle cancer alone. Poverty also limits access to treatments and resources and when health literacy is low, it is more difficult to manage care, e.g., understand medical instructions is harder. These speak to the importance of identifying and helping resource-deprived oncology subgroups.

Differential Diagnosis of Major Depression

Mood disorder due to a general medical condition is where the depression is judged to be a direct physiological effect of the medical condition, e.g., stroke and hypothyroidism. Hypoactive delirium is an important consideration in patients who are lethargic or labeled as “failure to thrive” or who seem depressed but are also disoriented (see “Discussion” later in this chapter).

A *substance-induced mood disorder* is where a drug of abuse or medication is causing depressive symptoms, e.g., alcohol or tobacco withdrawal. A number of medications have been associated with depression, but the strongest correlations are with steroids, interferon alpha, and taxanes. Smoking cessation predisposes an individual to a higher rate of major depression in the year subsequent to quitting [20].

Bipolar disorder is common and should be considered where there is a family history of bipolar or other affective disorders, or when depression does not respond to a standard 4-week antidepressant course.

Measuring and Screening for Depression

One measure for depression screening is the Patient Health Questionnaire-9 (PHQ-9) that has been widely validated in medical populations (Table 60.4) [21]. Items one (anhedonia) and two (depressed mood) must be positive in order to

Table 60.4 The PHQ-9

PHQ-9. Over the *last 2 weeks*, how often have you been bothered by any the following problems?

	Not at all	Several days	More than half of the days	Nearly every day
1. Little interest or pleasure in doing things	0	1	2	3
2. Feeling down, depressed, or hopeless	0	1	2	3
3. Trouble falling or staying asleep, or sleeping too much	0	1	2	3
4. Feeling tired or having little energy	0	1	2	3
5. Poor appetite or overeating	0	1	2	3
6. Feeling bad about yourself—or that you are a failure or have let yourself or your family down	0	1	2	3
7. Trouble concentrating on things, such as reading the newspaper or watching television	0	1	2	3
8. Moving or speaking so slowly that other people could have noticed. Or the opposite—being so fidgety or restless that you have been moving around a lot more than usual	0	1	2	3
9. Thoughts that you would be better off dead or of hurting yourself in some way	0	1	2	3

Total score___________ = Add columns_________ + ______________ + ______________

If you checked off *any* problems, how *difficult* have these problems made it for you to do your work, take care of things at home, or get along with other people?

Not difficult at all	Somewhat difficult	Very difficult	Extremely difficult
❍	❍	❍	❍

Interpretation of scoring: 0–4 none (below threshold of significance), 5–9 mild depression, 10–14 moderate depression, 15–19 moderately severe depression, 20–27 severe depression

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meet the depression threshold. In fact, Veterans Affairs hospitals in the USA use these first two items (known as the PHQ-2) to screen all of their patients. A score of ≥5 on the PHQ-9 represents significant depression. Thus the PHQ-9 can be used as a screening measure in oncology clinics and to assess depression severity, e.g., before and 3 weeks after starting an antidepressant. Translations into other languages such as Spanish, Chinese, and Russian are widely available online and are another useful resource for oncologists. If a response to item nine (thoughts of being better off dead) is positive, further questions about suicidality are necessary. Even use of a single question, “Are you depressed?” had a specificity and sensitivity of 1.00 for identifying depression in a palliative care cohort [22].

Anxiety, Depression, and Suicide

Cancer patients have twice the risk of suicide compared to the general population, and risk factors include depression, hopelessness, helplessness, loss of control, perceived burden to others, pain, cognitive function and delirium, poor social support, personality disorders, and existential distress [23].

While the association between depression and suicide is well known, more recently, there has been an increasing awareness that anxiety is another risk factor for suicide [24]. It seems likely that interactions between other vulnerabilities such as younger age, less social support, and specific anxiety subtypes (e.g., panic) might confer increased risk of suicidality [6]. Depression is associated with a greater desire for physician assisted suicide and, conversely, treating depression decreases the number of such requests.

Medical Workup for Depression

A thorough medical history and physical examination should consider vital signs, cardiovascular, neurological, gastrointestinal, and respiratory systems. Diagnostic tests may include laboratory examination (such as electrolytes, thyroid function tests, liver function tests, albumin, blood urea nitrogen, creatinine), electrocardiogram, brain imaging studies to exclude structural CNS lesions, electroencephalogram when seizure activity is suspected, and cerebrospinal fluid analysis (if CNS infection, subarachnoid hemorrhage, or leptomeningeal disease is suspected).

Psychopharmacological Treatments of Anxiety and Depression

Because psychological symptoms occur in approximately one-third of cancer patients, oncologists should have demonstrable competency treating their patients with antidepressants and antianxiety agents. Starting an antidepressant together with a benzodiazepine makes clinical sense where there is comorbid anxiety or prominent sleep disturbance, as the patient will experience some relief straight away. Adherence with antidepressants is also improved when they are prescribed together with benzodiazepines.

Although serotonin norepinephrine reuptake inhibitors (SNRIs) and selective serotonin reuptake inhibitors (SSRIs) and other such medications are called antidepressants, when taken regularly, they also have significant antianxiety effects; hence, the term antidepressant does not entirely match the function of the drug class. They might be better described as antidepressant–antianxiety agents.

Antidepressants

SNRIs, such as venlafaxine and duloxetine, and SSRIs, such as citalopram and sertraline, are considered first-line agents for treating depression in patients with comorbid medical conditions. One advantage of using an SNRI is that they have been found to be helpful in reducing neuropathic pain associated with diabetic neuropathy; however, SSRIs have no impact on pain pathways.

Antidepressant Titration Protocol

A good rule of thumb is to prescribe half of the lowest starting dose for 3–7 days to ensure that the medication is well tolerated. To illustrate, venlafaxine SR 37.5 mg is given for 1 week, after which it is increased to 75 mg daily. The dose of an antidepressant is reevaluated every 3–4 weeks by a clinical examination and measuring the PHQ-9 and GAD-7 for a 50% decrease in score. If there is no improvement, the dose is usually doubled, e.g., venlafaxine SR is increased from 75 to 150 mg/day after 1 month. If there is still no improvement after 2 months, the dose could be increased to 225 mg/day for a further month. If the depression still has not improved significantly by month three, the next step would be to (1) add an antidepressant from a different class (e.g., mirtazapine or bupropion), (2) switch antidepressants, or (3) augment with psychotherapy.

After symptoms remit (e.g., PHQ-9 score of <5) patients should continue the antidepressants for another 6–8 months to consolidate the remission. Discontinuing the antidepressant earlier increases the statistical chance of relapse. If depression is recurrent, the patient should consider continuing treatment for 2–5 years. It is not recommended that an antidepressant be stopped during the winter months due to the tendency of symptoms to worsen when they overlap with seasonal affective disorder. Spring or summer months are better times to wean antidepressants.

Side Effects of Antidepressants

SNRIs and SSRIs should be taken with food to minimize the most common initial side effect, transient nausea. Patients should be warned about this and told not to stop the medication if they experience nausea or other gastrointestinal symptoms because tolerance usually develops after a day or two.

A serotonergic withdrawal reaction occurs in one out of five patients when these medications are stopped suddenly. This presents as intense discomfort, and flu-like symptoms and electric-like shocks. Generally, it is prudent to tell patients that when antidepressants are stopped, the dose should be weaned gradually over 1–2 weeks. In the event of serotonin discontinuation syndrome, the antidepressant should be restarted and weaned even more slowly over the course of 4–6 weeks.

Sexual side effects can be bothersome and manifest as decreased libido in both sexes or delayed ejaculation in males. This is not, however, universal; many patients experience an improvement in their libido as the depression lightens.

Hyponatremia secondary to syndrome of inappropriate antidiuretic hormone has been recognized with SNRIs, SSRIs, and mirtazapine with a mean time to onset of 13 days (range 3–120) [25]. Those older than 65 are at particular risk.

There is an increased risk of gastrointestinal and uterine bleeding with SSRIs and SNRIs. The mechanism is believed to be via depletion of platelet serotonin which impedes coagulation in vulnerable patients, e.g., those taking nonsteroidal anti-inflammatories.

Some antidepressants have specific side effects: Mirtazapine is a sedating antidepressant and some patients complain of feeling groggy in the morning. Patients with a prominent sleep disturbance may, however, appreciate a combination antidepressant-sleeping tablet. Mirtazapine may also cause weight gain and, anecdotally, this can be helpful to some oncology patients.

Bupropion, unique among antidepressants, works via a dopaminergic mechanism. It is particularly useful as an aid to nicotine cessation where it increases the chance of successful quitting. It may, however, be too activating for some patients who complain of tremors or feeling wired. It may also reduce the seizure threshold, requiring caution in patients with a seizure diathesis.

Venlafaxine may cause a dose-related increase in hypertension although doses up to 225 mg are usually safe.

Cytochrome P450 drug–drug interactions can also occur. For example fluconazole, commonly used in the transplant setting, inhibits CYP2C19, the enzyme that metabolizes the antidepressant citalopram [26]. The result may be serotonin toxicity, a syndrome of neuromuscular hyperactivity (tremor, clonus, myoclonus, hyperreflexia, and the advanced stage pyramidal rigidity), autonomic hyperactivity (diaphoresis, fever, tachycardia, tachypnea, and mydriasis), and altered mental status (agitation and confusion in advanced stages). Serotonin toxicity is reversible upon cessation of the triggering medication. An identical serotonin syndrome occurs in overdoses of SSRIs and SNRIs. Mirtazapine has not been associated with serotonin toxicity.

Paroxetine and fluoxetine were, until recently, prescribed for postmenopausal hot flashes. However, they inhibit CYP2D6 which reduces the metabolism of tamoxifen to its active metabolite, endotamoxifen. The result is an increase in breast cancer relapse [27]. For this reason venlafaxine is preferred for the treatment of hot flashes in patients receiving tamoxifen.

Tricyclic antidepressants may result in orthostatic hypotension, anticholinergic side effects, and quinidine-like effects. They are dangerous in overdose. Monoamine oxidase inhibitors require a tyramine-free diet. Hence, these drugs are now considered third- or fourth-line agents.

Benzodiazepines

Although there are a few controlled trials of benzodiazepines for anxious cancer patients they are widely used from diagnosis to survivorship and from acute hospitalization to terminally ill care. They can be administered in a variety of ways, which is an important consideration in medical settings where oral intake may be compromised (e.g., lorazepam: orally, intramuscularly, intravenously; clonazepam: orally, orally disintegrating tablet). Longer acting benzodiazepines, such as clonazepam or extended release alprazolam, may provide more persistent relief of anxiety symptoms, but patients often report satisfaction from short-acting alprazolam because of its quick onset and offset. They find it helpful to reduce worry prior to brief procedures such as MRI scans, radiation, bone marrow aspirations, or when they are in the physician's waiting room, nervously awaiting the results of an important test.

In addition to treating anxiety, benzodiazepines are also useful for insomnia, nausea, vomiting, and panic attacks. Both lorazepam and alprazolam have been shown to reduce postchemotherapy as well as anticipatory nausea and vomiting [28, 29]. It is also hypothesized that given before chemotherapy or a procedure, benzodiazepines may reduce conditioned aversion responses due to their amnesic properties [28, 30].

The Society of Critical Care Medicine guidelines for the management of anxiety in critically ill adult patients recommend the use of lorazepam for prolonged (i.e., more than 24 h) treatment of anxiety in critically ill patients [31]. Lorazepam is probably the most widely used benzodiazepine in acute cancer settings.

How do benzodiazepines compare to behavioral interventions for anxiety? Their efficacy is similar. For instance, one randomized trial of alprazolam versus progressive muscle relaxation found that both treatments resulted in a significant reduction in anxiety symptoms, but the effect of alprazolam was faster [32]. For the oncologist, this means that there are efficacious behavioral options for patients who wish to avoid antianxiety drugs.

Side Effects of Benzodiazepines

Sedation, drowsiness, and ataxia, the commonest side effects of benzodiazepines, increase the risk of falling in elderly patients.

Another big drawback of benzodiazepines is that they increase the risk of delirium, especially in the elderly and in those with multiple comorbidities. It is prudent to stop benzodiazepines when a patient becomes delirious. Memory difficulty, sexual dysfunction, and incontinence are encountered less frequently.

Impaired hepatic function or drugs that compete for the liver enzymes that metabolize benzodiazepines (e.g., erythromycin, estrogen, and isoniazid) may necessitate lower doses of benzodiazepines to minimize side effects. Lorazepam, oxazepam, and temazepam are preferred if hepatic function is impaired because they are metabolized by conjugation with glucuronic acid and have no active metabolites [30, 33].

Benzodiazepines have a dose-related effect on the respiratory center, but when their use is carefully monitored, they may reduce anxiety and improve the respiratory function among patients with anxiety due to dyspnea.

Antihistaminergic agents are occasionally used in anxious patients with severely compromised pulmonary function for whom benzodiazepines are contraindicated. Antihistamines, however, do not have anxiolytic properties. Their sedative effects most likely result in a sense of anxiety relief. The anticholinergic effects of these medications must be monitored carefully as they increase delirium risk.

Psychostimulants

Methylphenidate and dextroamphetamine are often used as energizing agents in oncology patients who are depressed, fatigued, or both. Evidence for their efficacy comes from open labeled trials, where 4/5 studies found them to be effective but, unfortunately, there are currently no placebo controlled studies [34]. Nevertheless, for patients who are depressed, fatigued, suffer from narcotic-induced sedation, or have difficulties concentrating, a stimulant can be helpful. Low doses are used initially, e.g., methylphenidate 5 mg at breakfast and 5 mg at lunchtime. This can be titrated up to 10–20 mg twice daily until some improvement in fatigue or function occurs. Stimulants should be used cautiously in patients with cardiac problems as tachycardia is a potential side effect. They are thought to have a low abuse potential in oncology settings.

Modafinil is a newer wakefulness promoting agent that lacks abuse potential and, although there are no studies of its efficacy in depressed cancer patients, it is anecdotally reported to be helpful. The starting dose is 50–100 mg. Lack of insurance reimbursement for this off-label use renders it an expensive option for many patients.

Psychotherapy

The following section focuses primarily on evidence-based psychotherapies. If possible, oncologists should refer their patients to therapists who employ such techniques. Cancer centers should recruit therapists with competency in these psychotherapies over “eclectic” practitioners. Nevertheless, treatment should be tailored to the patient’s and family’s needs rather than assuming that one solution will fit all. Comorbidity such as intercurrent alcohol, drug, or nicotine dependence should be addressed first and foremost.

Psychoeducation

This provides information (verbal, written, visual, or a combination) to help people learn ways of mastering a novel situation. To illustrate, an orientation to the stem cell transplant ward can demystify what lies ahead and help patients and families plan for the transplant. Acquisition of new knowledge is essential to navigate an often foreign medical system; unfamiliarity increases anxiety and uncertainty. A related concept is that of patient navigators who provide barrier focused assistance to ensure completion of a discrete episode of cancer-related care.

Typical of many, one psychoeducation RCT involved an orientation tour of the clinic, written resources for coping (e.g., support groups), and a question and answer meeting with a counselor; anxiety was significantly less at follow-up [35]. A meta-analysis of 116 psychoeducational studies [36] reported a moderate effect size but another failed to show an effect [37].

Cognitive Therapy

This is considered by many to be the most effective psychotherapy for anxiety and depression. Cognitive therapy is based on the principle that it is not a situation that results in

Table 60.5 Maladaptive cancer cognitions that are associated with anxiety

Cancer-related automatic thought	Consequence
I will die in my sleep	Insomnia, nocturnal anxiety, hypervigilance
Chemotherapies are toxic, poisonous chemicals	By over-generalization of all anticancer treatments into the drain cleaner category, well-tolerated regimens and effective symptom management are negated. Patients may flee to "natural" medicine cures.
I *must* think positively!	This is called the "tyranny of positive thinking." Everyone tells the patients to "think positive" but, invariably, a realistic or pessimistic thought crosses the cancer patient's mind. They then feel guilty, feeding into despair and depression.
Cancer *means* death	The term cancer is over-generalized. In fact cancer is a heterogeneous, nonspecific term. The majority of cancer patients are cured. This belief often leads to paralysis and demoralization.

an emotion but rather the preceding automatic thought [38]. To illustrate, one person undergoing a CT scan may think, "I know that the pain is cancer," and feel sad. The next person through the scanner may think, "I am glad that I will finally have an explanation for the pain," and feel relief.

Typical maladaptive cancer-related automatic thoughts associated with depression and anxiety are listed in Table 60.5.

One meta-analysis found a small but significant effect size of cognitive therapy; another in cancer survivors found a larger effect size [37]. Data in nonmedical populations support the efficacy of cognitive therapy, with large to moderate effect sizes reported [39].

Behavioral Activation

Consider two equally depressed patients with identical hematological malignancies. One remains in bed, alone in the dark. The other is forcing himself to walk around the nurses' station. Who will feel more depressed? It is reasonable to assume that the withdrawn, isolated person will feel more depressed and the activated patient less so. This is the basis of Behavioral Activation (BA): that activity and engagement improve mood while withdrawal and avoidance maintain depression. BA strategies include scheduling, self-monitoring, rating the degree of pleasure and achievement during daily activities, and exploring alternative behaviors with techniques such as role-playing. In one (nononcological) RCT, BA was as efficacious as antidepressants in severe major depression [40].

Problem-Solving Therapy

This therapy is based on the paralysis consequent to dealing with multiple health, employment, financial, and family problems simultaneously. Through this therapy, patients learn to define the problem, brainstorm possible options, evaluate potential solutions, implement solutions, monitor the degree of their success, and make adjustments to the solutions. A family member can also be incorporated as a coach. Examples of target ideations in problem-solving therapy are a patient who may erroneously believe that "All of my problems are due to the cancer." [The cancer is probably not to blame for every problem that s/he has], or an individual who expresses the belief that *"No-one* can help." [Many patients are helped.] In one RCT of oncology patients, problem-solving effectively reduced patients' distress [41].

Behavioral Therapies

Behavioral therapies are among the most widely used psychosocial treatments offered to cancer patients. They are efficacious for anxiety, the "here and now" distress associated with cancer treatment side effects and anticipatory nausea but constitute an array of interventions.

In *distraction*, attention is diverted away from the distressing stimulus, e.g., wiggling toes during venipuncture, guided imagery, storytelling, or video games. *Systematic desensitization* involves gradual reexposure to a feared stimulus where relaxation is combined with gradual visualization of cues associated with the trigger. The end point is habituation to the feared stimulus so that the aversive reaction is contained, e.g., an aversive reaction to eating after chemotherapy induced vomiting. *Hypnosis* is where, via relaxation techniques, the patient learns to focus attention on thoughts and feelings unrelated to the source of distress, at which point the clinician introduces suggestions for calm and well-being, reducing anxiety. *Relaxation training* uses techniques such as progressive muscle relaxation (systematic tensing and releasing of muscle groups), meditation, or deep breathing. Slowing down breathing is a key ingredient in relaxation training because hyperventilation increases panic. Relaxation training should be used with caution in patients with a history of sexual or physical abuse, for whom relaxation might be perceived as being dangerous. Relaxation training can also be self-administered. One study effectively used print and audiovisual media to instruct patients in paced abdominal breathing, progressive muscle relaxation with guided imagery, and coping self-statements, with only 10 min of instruction from the clinician [42].

Support Groups

Cancer support groups have a high degree of user satisfaction but heterogeneity in methodologies makes it hard to compare their effects on anxiety and depression [43]. Online support groups such the Cancer Support Community (https://www.cancersupportcommunity.org/) are now well established and bridge physical barriers and bricks and mortar costs. "Supportive expressive group therapy" has been shown to help depression or helplessness/hopelessness [44, 45]. Support groups constitute an important resource for oncologists.

Psychotherapy vs. Psychopharmacology vs. Combined Treatment

In meta-analyses (conducted on nononcology patients), pharmacotherapy for anxiety and depression works faster than psychotherapy, but longer term outcomes are more robust for psychotherapy [46, 47], i.e., talking through your problems has lasting benefits. Although combined cognitive and pharmacotherapy does not have a clear advantage in meta-analyses, these data are not without controversy [46, 47]. In practice, combined treatment seems to be the most beneficial [30, 48, 49]. Interestingly, compared to medication alone, combined therapy patients report fewer side effects of medications [50]. Psycho-oncology, due to the urgency of its context, also favors treatments that work faster; rapid symptom relief may facilitate more timely, definitive cancer treatment. For patients who are too sick to enter a talk therapy or who are not psychologically minded, timely psychopharmacological treatment is a valuable tool in their recovery.

Delirium

Delirium, or confusion, is a complex neuropsychiatric condition that manifests in one-third to one-half of inpatients; terminally ill patients are affected at even higher rates. Acute delirium is a medical emergency because it reflects brain malfunctioning due to an underlying systemic condition that demands immediate treatment. The term derives from the Latin "De," which means away from and "lira" which is a furrow in a field and, thus, literally means going off the plowed track.

Pathophysiology

Generalized cerebral dysfunction is reflected in the electroencephalogram which shows diffuse slowing of the dominant rhythm and functional imaging that shows generalized cortical hypoperfusion, both of which normalize with resolution of the delirium. Multiple neurotransmitters are implicated, but hypoactivity of the cholinergic system is seen as a common pathway. This is the basis of using neuroleptics to treat delirium symptoms—as dopamine antagonists, they increase cholinergic activity. Gamma aminobutyric acid (GABA) excess (hepatic encephalopathy, propofol, benzodiazepines) or deficiency (alcohol withdrawal) are other delirium pathways. More recently, noradrenergic hyperactivity has been viewed as an important common pathway [51].

Risk Factors

Delirium is more likely in individuals with the following risk factors: age > 75, preexisting cognitive impairment, sleep deprivation, hypoxia, metabolic abnormalities, alcohol or drug abuse, visual impairment (increases risk 3.5 times but not auditory impairment), severity of underlying illnesses, restraints, catheters, leg compression devices, casts, ventilation, and polypharmacy (especially those medications with high anticholinergic potential).

Clinical Features

Delirium is characterized by an abrupt onset of altered consciousness (alertness) with difficulty in focusing, sustaining, or shifting attention. Typically symptoms fluctuate throughout the day and night. Mood is impaired and it is not uncommon for these patients to be referred to psychiatry for evaluation of depression because they seem depressed or withdrawn. Cognitive problems such as disorientation, memory loss, incoherent or rambling speech, dysnomia, dysgraphia, and perseveration are seen. Perceptional problems such as hallucinations and delusions are common and are disturbing to patients and their families alike. Psychomotor activity may be increased in agitated delirium but lethargic, hypoactive delirium is more frequent. Neurological signs such as asterixis, tremor, frontal release, myoclonus, and altered muscle tone are common [14].

Course

Delirium lasts on average 10–12 days (range 1–8 weeks), but 15% of cases last more than 30 days. Although delirium was originally seen as completely reversible, it is now recognized that, in 20%, there are signs of dementia or cognitive decline 1 year later. In cases of recurrent delirium, cognitive decline is even more pronounced and mortality is greater. Delirium results in a prolonged length of hospital stay, greater medical expenses, and secondary complications such

as falls, nosocomial infections, and pressure sores. Terminal delirium is important to recognize as a harbinger of approaching death.

Mortality

The 6-month mortality in ICU patients is 34% in delirious patients vs. 15% in nondelirious, 8% in delirious hospitalized patients vs. 1% in nondelirious hospitalized patients, and 11% in elderly delirious patients vs. 3% in nondelirious patients [51].

Management

Delirium, like anemia, is not a diagnosis but rather a clinical syndrome, therefore its treatment addresses the underlying cause (Table 60.6). Often the triggers of delirium are multifactorial with medications being well-known iatrogenic culprits. Narcotics are common causes of delirium in oncology settings and, of the narcotics, meperidine has the greatest propensity for delirium. Antihistamines, benzodiazepines, and steroids are also commonly implicated.

It is often not appreciated that a variety of common medications have an anticholinergic load including furosemide, digoxin, theophylline, prednisone, and nifedipine. Anticholinergic activity correlates directly with delirium [52]. Elderly patients who take multiple drugs with anticholinergic burden have measurably decreased physical strength and worse functional ability [53]. As the cholinergic load of drugs is not usually available on standard pharmacy databases, common sense wisdom suggests stopping all unnecessary medications in delirious patients, including antidepressants.

Table 60.6 Clinical risk factors for delirium

Infection: UTI, pneumonia, sepsis, meningitis, infected line, or device
Cardiac: acute infarction, hypotension/hypoperfusion, arrhythmia, congestive heart failure
CNS: stroke, normal pressure hydrocephalus, bleed, trauma
Renal: failure
Hepatic: failure
Respiratory: Hypoxia, pulmonary embolus, respiratory failure
Nutritional: malnutrition, thiamine/Wernicke's encephalopathy, dehydration
Endocrine: hyper/hypothyroidism, hyper/hypoglycemia, hyper/hypokalemia, hyper/hyponatremia, cortisol deficiency, or excess states
Sleep: deprivation, over sedation
Environmental: Hyper/hypothermia, trauma/surgery, fractures
Substance withdrawal, intoxication, heavy metal poisoning
Pain: poorly controlled

Nonpharmacological treatments include normalizing night/day, sleep/wake cycles (an ICU patient gets less than 2 h sleep/night), avoiding restraints, ensuring the calming presence of family, 1:1 nursing, and precautions against falls.

Intravenous haloperidol is considered the gold standard for targeting agitation, hallucinations, and for reducing the overall length of the delirium episode [54]. Oral haloperidol should be avoided because of its propensity to cause extrapyramidal symptoms. A dose of 0.5–2 mg IV can be administered every 2–6 h, or less frequently if necessary. Alternatively, it can be given as a continuous infusion. The maximum daily dose of 20 mg/day is usually considered adequate, although, it is safe to increase the dose as long as the QTc is not prolonged. A QTc > 480–500 ms or a 25% increase from baseline is of concern. QTc prolongation causing Torsades de Pointes and sudden death is the most dangerous side effect of this class in medical settings but the incidence of neuroleptic malignant syndrome, manifested by stiffness, fever, and a raised CPK, is less common.

Intravenous chlorpromazine 12–50 mg every 4 h and oral or orally disintegrating olanzapine 2.5–5 mg every 12–24 h are other common alternatives. Clinicians prescribing atypical antipsychotics such as olanzapine should be familiar with FDA black box warnings for increased risk of mortality and cerebrovascular disease in the elderly [55]. Olanzapine has recently been shown to be helpful for delirium prophylaxis when 10 mg is given immediately prior to surgery and immediately after [56].

Dexmedetomidine has been shown to significantly reduce the rate of postoperative delirium after cardiac surgery and represents an exciting future direction, representing a therapy targeted to the norepinephrine common pathway [57].

Benzodiazepines should generally be avoided as they can worsen delirium, the only exception being alcohol withdrawal, where benzodiazepines are used as a treatment and antipsychotics are not usually given. Flumazenil, a benzodiazepine antagonist, has been shown to be helpful for hepatic encephalopathy, where GABAminergic excess is postulated.

In terminal delirium, the goal is comfort and benzodiazepines or other sedating agents such as barbiturates, which are usually avoided in delirium, can be given for terminal agitation.

Hematopoietic Stem Cell Transplant

Hematopoietic stem cell transplant (HSCT) offers a chance for cure but at the risk of morbidity and mortality. After the transplant, patients and their families must deal with three types of problems: physical, psychological, and community reintegration [58].

Physical problems after a HSCT include fatigue, physical disability (e.g., Graft Versus Host Disease (GVHD),

complications of steroids, hair loss), complications (e.g., shingles, sepsis), eating problems, nausea, vomiting, immune restrictions (e.g., limitations in gardening, being in crowds), difficulties with sex, and infertility. Even if patients survive the acute phase, they still must be wary of the need to manage long-term sequelae such as chronic GVHD, osteoporosis, cataracts, hypothyroidism, respiratory problems, and secondary cancers.

Psychological problems include anxiety and depression colored by a pervasive caution and fear of relapse or infection. Due to immune restrictions, isolation often contributes to loneliness and boredom. Patients may have survivor's guilt that fellow transplant patients did not make it or true bereavement at having lost "hospital" friends. They may be angry at the world, asking, why me?

After the dislocating HSCT, a further challenge is reintegration back into family life, working, social and sporting environments. Relationships are stressed especially where caregivers are vulnerable to burnout. Intimacy may be altered by the prolonged illness, neuropathy, or dry mucous membranes. Not infrequently, couples have marital conflict or separate. Resuming family roles with the expectation that life will continue where it left off before the transplant is common but unrealistic. Stigma is real and patients may encounter work discrimination or feel tainted. Infertility complicates attempts to date and form relationships and the question of what to disclose, and when, is always worrying. Stress resonates through the whole family network; often the whole family suffers financially as the cost of medical expenses balloons.

Therefore, it is not an exaggeration to say that full recovery from a myeloablative HSCT is a 3–5 year process. At 3 and 5 years, about 20% of these patients still have major physical limitations. In addition, 42% and 13% still report significant distress at the 3 and 5 year marks [59].

Are physical outcomes predicted by whether the transplant is autologous or allogeneic or whether the patient develops chronic GVHD? One would think so but this is not necessarily the case. Depression, radiation pretransplant, and higher medical risk but not the transplant type or chronic GVHD, predict physical outcomes [59].

Chronic GVHD is, however, associated with greater depression but depression is not predicted by the transplant type or high vs. low medical risk. Nevertheless, depression does resolve faster in autologous transplant patients. Overall, 22% of HSCT patients will develop major depression at one point or another during the transplant, supporting the need for skills to manage and treat depression. By 5 years, 84% of survivors without recurrence, who worked prior to the HSCT, have returned to full-time employment [59].

Finding a donor can be stressful. If a sibling is a donor but the patient ultimately dies, that sibling may feel responsible or attribute the death to his/her own shortcomings. Where there is sibling rivalry or conflict, willingness to donate may not be a given. The matching process may also reveal that a parent or sibling is not biologically related to the patient and this may be unwelcome knowledge at a sensitive time.

Psychiatrists and psychologists perform an important role in screening patients for suitability prior to transplant and all HSCT programs should consider the benefits of this type of screening program. Such screening programs are already the norm in solid organ transplantation programs. In pretransplant evaluations, transplant psychiatrists (or psychologists) must make it clear that they represent the transplant team and that the report generated will be sent to the transplant oncologist and become part of the medical record. It is equally important to state that the assessment is not a pass/fail exam but rather aims to identify psychological or family problems as early as possible so as to resolve difficulties effectively. Thus, the stance is one of teamwork, problem-solving, and anticipation.

Patients should be screened for current depression, anxiety, and substance use (alcohol, cannabis, nicotine, stimulants, narcotics, etc.). A detailed history should probe for past depression, anxiety, substance use, and other psychiatric issues, as the past is predictive of the future. Personality traits should be considered but in reality, only low functioning borderline or other personality disorders, for whom adherence has been demonstrably poor in the past, represent contraindications to transplant. This is because poor or inconsistent adherence represents a clear danger to patients who require immunosuppressive treatments posttransplant. Schizophrenia and bipolar disorder are not contraindications to transplant and many such patients have been successfully transplanted. Family cohesion, conflict, and supports are also an important part of the pretransplant psychiatric assessment.

A number of behavioral strategies can be useful to patients entering the transplant process. Anticipation leads to better planning and preparation. A tour of the transplant floor is helpful to demystify the aura of the transplant and to promote future-oriented planning. For example, the patients should consider how they will occupy their time productively during the long admission. Hobbies can be adapted to the hospital setting. Video games such as "Wii Fit" can be brought to hospital. Early involvement of physical therapists is beneficial. Modified exercise and stretching promotes an early expectation of rehabilitation and wellness. The hospital can provide exercise bicycles that remain in the room permanently. Exercise bands can be used to maintain muscle tone. Integrative medicine and music therapy can be very helpful in promoting wellness, and hope, counteracting potential patient passivity.

Planning for the best, worst, and most likely outcomes facilitates an open dialogue for discussing the possibility of death and advance care directives. Health care agents should be involved in this discussion.

Patients often experience the transplant infusion as an anticlimax. This point can be used to educate patients that, technically speaking, the transplant will not be dramatically different from the various infusions and treatments that they have received to date.

Discharge home can paradoxically trigger depression—this is known as the "vacuum effect." Patients transiently feel lost and abandoned after weeks of intensive inpatient treatment. When their doctor instructs them to return to the hospital in a week's time, they have difficulty adjusting to life outside the cocoon. Usually, educating the patient and the family about the "vacuum effect" is all that is required but antidepressants may be helpful if major depression develops.

Physician burnout is an important issue as transplant physicians are extremely hard working and conscientious. Treatments are prolonged and fatiguing. Mortality is high. Furthermore, physicians often feel pressured to accept patients with a low likelihood of survival, but few alternative oncology treatment options. One transplant physician dubbed these transplants, which are likely doomed to failure, as "Hail Mary transplants." Burnout is discussed in further detail later in this chapter.

Future developments will likely see an increase in outpatient transplant volumes, widened indications, older patients, and newer technologies, e.g., chord transplants, all of which have challenges for psycho-oncology.

Neurocognitive Effects of Cancer Treatments

Once referred to colorfully as "chemo brain," it is now appreciated that a variety of anticancer treatments can cause neurocognitive damage. Typically these patients complain of difficulty with executive functioning, such as multitasking, and diagnosis is made via formal neurocognitive testing. Possible mechanisms include direct neurotoxicity, an immunologic inflammatory response, or microvascular injury. Neurocognitive changes can last from 2 months to 3–10 years or more. Particular vulnerability is seen in carriers of the Alzheimer's susceptibility gene, Apolipoprotein (APOE) [60].

Communication Skill Training

Patients generally recall only about 25% of the data that oncologists consider important and, furthermore, poor information provision increases rates of depression, which is understandable because not knowing what is going on can be demoralizing.

Communication skills training can help physicians to more effectively break bad news, discuss prognosis, manage angry patients (or families), discuss unanticipated adverse events, end-of-life goals of care, Allow Natural Death (Do-Not-Resuscitate) directives, and the withdrawal of life-extending care. Knowing how to effectively conduct a family meeting is an essential skill that is not taught in most training programs. Programs such as Oncotalk or Memorial Sloan-Kettering Cancer Center's Comskil program use role-playing with simulated standardized patients, which has been found to be an effective training method. All oncology programs should consider the value of adding communication training to their educational program.

Burnout and Compassion Fatigue

Burnout is a well-defined and measurable psychological triad of emotional exhaustion, depersonalization (cynicism towards patients), and a sense of reduced personal accomplishment (inefficiency) that is common in oncology settings [61, 62]. Ramirez described a 28% prevalence of burnout among cancer physicians, a rate that is similar in nononcology physicians [63].

Burnout at work can be moderated by an oncologist's personal happiness, family cohesion, personal psychiatric problems (such as depression), physical fitness, and creativity. Work difficulties in combination with problems outside of work can increase the risk of overwhelming the individual and precipitate burnout. Younger (age < 55), single and female physicians are more likely to develop burnout. The Physician Work Life Study found that women may be 1.6 times more likely to report burnout than males. Additionally, for every 5 h worked over the typical 40 h per week, the odds of burnout increased 12–15% for female physicians, but not for males [64]. In other words, working reasonable hours is important if a woman is expected to juggle both an oncology career and a family. In these studies, career support from a spouse provided a buffer for female physicians against burnout.

Unfortunately, there is evidence that the mental health of physicians has worsened over time. In 2002, compared to 1994, physician psychiatric morbidity had increased from 27 to 32% and burnout from 32 to 41%, with worse results in physicians working in oncology compared to radiology or gastroenterology [65]. Worsening burnout rates may also reflect an overburdened health system.

As recognition of this vulnerability of physician burnout, the Joint Commission on Accreditation of Healthcare Organizations in the USA has mandated a distinct process that deals with physician well-being, separate from the disciplinary processes.

Burnout can be addressed either prophylactically or remedially; a tailored approach is probably most effective. Meta-analytical data provides preliminary support for individual-focused and organization-focused burnout interventions [65].

Collaborative Care

Collaborative Care is a new population-based approach which targets the patient, clinician, and system of care. Its aim is to measurably improve psychological outcomes and, in so doing to, improve medical outcomes and utilization of services. The latter is important in Accountable Care Organizations where fee-for-service is replaced by capitated arrangements providing an incentive to keep patients out of hospital, reduce emergency department utilization and length of stay.

Care managers (usually nurses or social workers) are supervised by a psychiatrist and, in so doing, act as physician extenders embedded into primary care or oncology settings. Thus, mental health is integrated into medical care. It also addresses the problem of access to psychiatric care which has long frustrated oncologists.

Collaborative care has shown that by treating depression and teaching patients to better manage chronic illness, cardiovascular and diabetes outcomes are improved [66].

A large multisite trial of collaborative care for depression in cancer patients used nurses to deliver up to 10 sessions of treatment comprised of psychoeducation, problem-solving, behavioral activation, and symptom monitoring, followed by 8 months of symptom monitoring with additional sessions as needed [67]. Depression was significantly improved in the collaborative care group as compared to treatment-as-usual (62% vs. 17%) and there were also greater improvements in anxiety, pain, fatigue, functional ability, quality of life, and perceived quality of care.

Conclusion

Because psychosocial problems are so prevalent in patients with hematological malignancies, oncologists should show demonstrable competency in treating depression, anxiety, delirium, and other such issues. Psychosocial oncology services, preferably integrated with palliative care, should be developed as it is almost impossible for one clinician to meet complex medical and psychosocial challenges alone. A multidisciplinary team approach is required and Collaborative Care is a new way to organize delivery of care effectively. Communication skills training has received increasing attention as a way for oncologists to improve their ability to manage complex scenarios or cancer decision-making more effectively. Finally, burnt-out oncologists represent a burden to their own personal health and happiness, to their families, and to their patients and colleagues. First and foremost, look after yourself!

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Index

A
ABH-mismatched platelets, 1162
Abnormal localization of immature precursor (ALIP), 225, 495
Abnormal promyelocyte, 207
Abnormal proteins
  amyloidosis, 1271–1272
  dysproteinemias, 1270–1271
ABO antibodies, 1143
ABO incompatibility transplants, 1221
ABO incompatible stem cell transplantation, 1134
ABO system, 1221–1222
ABO-identical *vs.* nonidentical platelets, 1161
ABO-mismatched platelet transfusions, 1161
Absolute lymphocyte count (ALC), 117–118
Acetylation, of histones, 413
Acid citrate dextrose (ACD), 376, 1130
aCML *See* Atypical chronic myeloid leukemia (aCML)
ACP-196 (Acalabrutinib), 127
Acquired immunodeficiency syndrome (AIDS), 711, 755, 976, 1081
  non-Hodgkin lymphoma, 765
Acquired von Willebrand disease (VWD)
  desmopressin, 1273
  diffuse oozing, 1272
Activated B-cell-DLBCL (ABC-DLBCL), 831
Activated B-cell-like (ABC) groups, 790
Activated protein C (APC) resistance, 1266
Activated signaling, 167–168
Activation-induced cytidine deaminase (AID), 833
Activation-induced deaminase (AID), 35
Active immunization, 1058
Acute basophilic leukemia, 219
Acute B lymphoblastic leukemia, 203
Acute erythroid leukemia, 218, 219
Acute graft-versus-host disease
  allogeneic HCT, 1226
  cytotoxic chemo- or chemoradiation therapy, 1227
  donor-derived T-cells, 1226
Acute hemolytic transfusion reactions, 1146
Acute leukemia, 181, 182, 184–187, 199, 1039, 1070, 1082, 1153
  acetaminophen for, 187
  after CD19-targeted therapy, 256
  of ambiguous lineage, 221, 240
  antigen and therapy, 252
  bacterial infections, 1041
  birth weight, 183
  BMI and diet, 187
  breast-feeding, 183
  characteristics, 179–180
  chemotherapy, 184, 185
  cigarette smoking, 187
  classification, 197, 199, 362
  combination chemotherapy, 155
  cytogenetics, 281–282
  definition, 197
  diagnosis, 197, 199
  diet and vitamin supplement, 185
  environmental exposure
    chemicals, 185
    ionizing radiation, 184, 186
    nonionizing radiation, 184
  evaluation of remission, 221
  familial aggregation and genetic syndrome, 187–188
  fungal infections, 1042
  genetic susceptibility, 188
  history, 155–158
  in vivo therapy with monoclonal antibodies, 252
  maternal diet and vitamin supplement, 182–183
  medical conditions and treatment, 181
  mode of birth, 183
  NSAID for, 187
  occupational exposure
    benzene, 186
    farming and pesticide exposure, 186–187
    radiation, 186
  parental alcohol consumption, 182
  parental chemical/pesticide exposure, 182
  parental cigarette smoking, 182
  physical activity, 187
  radiation, 184–186
  radiation exposure
    environmental ionizing, 181–182
    medical, 181
    parental occupational, 181–182
  reproductive history, 181
  risk factors for, 180–181
  treatment, 183–184
Acute leukemia, immunobiology, 237–247
  antigen profiles for leukemia fusion genes, 241
    cryptic t(12;21)(p13;q22)-*TEL(ETV6)/AML1(RUNX1)*^{POS} B-Lineage ALL, 247
    Inv(7)(p15q34) and t(7;7)(p15;q34)-*TRB@/HOXA*, 247
    t(15;17)(q22;q12)-*PML/RARα*^{POS} APL, 242–243
    t(4;11)(q22;q23)-*KMT2A (former MLL)/AF4*^{pos} B-Lineage ALL, 246–247
    t(8;21)(q22;q22)-*RUNX1/RUNX1T1*, 243–244
    t(9;22)(q34;q11)-*BCR/ABL*^{pos} ALL, 244–246
  immunophenotype
    vs. genetic lesion, 241
    vs. morphology, 239–241

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Acute leukemogenesis, 161
Acute lymphoblastic leukemia (ALL), 24, 52, 62, 161, 163, 179, 197, 200, 203, 249, 359, 377, 776, 976, 1009, 1039, 1057, 1131, 1186, 1248, 1254, 1255
cytochemistry, 200–202
cytogenetics, 293–296
aberrations in, 291–292
adverse prognosis, 293–294
chromosome numbers in, 290
Philadelphia Chromosome (Ph+) and Philadelphia-like, 294
recurrent deletions and single-chromosome aneusomies, 296
uncertain prognostic significance, 295–296
genetics, 202–203
immunohistochemistry, 202
incidence, 180
morphologic features, 200
relative risk for, 180
Acute lymphoblastic leukemia (ALL), adult
adolescents and young adults with, 350
chemotherapy, 344–345
clinical approach and laboratory investigation, 338
clinical features, 337, 338, 342
CNS therapy
established CNS disease, 347
prophylactic, 347
complete remission, 345–346
consolidation therapy, 346
cytogenetics and molecular genetics, 342–343
diagnosis, 339
diagnostic procedures, 338
bone marrow examination, 339
cerebrospinal fluid examination, 339–340
laboratory investigations, 339
peripheral blood examination, 338–339
differential diagnosis, 340
hematopoietic growth factor, 344
hemorrhage, 344
immunological subtype, 342
immunological, morphological, cytogenetic, and molecular characterization, 341
induction therapy, 346
initial treatment, 345
integrated risk classification, 353–354
long-term follow-up and late effects, 354
maintenance therapy, 346–347
morphology and cytochemistry, 340
B-lineage ALL, 341
immunophenotype, 340–341
myeloid antigen-positive ALL, 342
T-lineage ALL, 342
MRD detection, 343–344
older patients with, 349
prognostic factors, 349
age, 349
for risk stratification, 353
MRD, 353
relapsed/refractory, treatment, 354
remission induction, 345
stem cell transplantation, 347–349
treatment response and MRD, 352–353
white blood cell count, 350–352
Acute lymphoblastic leukemia (ALL), childhood
chromosomal abnormalities, 311, 312, 315, 316
chromosomal translocations and gains/deletions
BCR-ABL1-like subtype, IKZF1 deletions, and CRLF2 overexpression, 312–313
BCR-ABL1-positive ALL, 312
ETV6-RUNX1 fusion, 312
KMT2A (MLL) gene rearrangement, 313
TCF3 rearrangement, 313
classification, 311
clinical and laboratory features at diagnosis, 308
clinical trials, 318
cytogenetics and molecular genetics, 311
diagnostic classification
immunophenotype, 309–311
morphology and cytochemistry, 309
differential diagnosis, 309
early T-cell precursor (ETP), 314
genetic lesion, 315
genetic subtypes of T-cell ALL, 314
hypodiploidy, 311, 312
immunophenotypic subgroups, 310
incidence, 307
intrachromosomal amplification of chromosome 21, 314
predisposing factors, 307–308
prognostic factors, 314–315
age, 315
CNS disease at diagnosis, 315–316
Down syndrome, 317
gender, 317
immunophenotype, 315
leukocyte count, 315
minimal residual disease, 316–317
race and ethnicity, 317
response to therapy, 316
treatment adherence, 317
relapse, 321–322
isolated extramedullary, 322
marrow, 322
treatment, 318
cardiac late effects, 321
CNS-directed therapy, 320
continuation (maintenance), 319–320
historical background, 317–318
neurocognitive late effects, 320–321
post-induction consolidation, 319
remission induction, 318–319
risk-adapted therapy, 318
second malignant neoplasm, 321
skeletal toxicities, 321
Acute lymphoid leukemia (ALL)
Acute lymphoblastic leukemia (ALL)
Acute megakaryoblastic leukemia (AMKL), 197, 211, 218
cytogenetics, 288–289
Acute monocytic leukemia (AMOL), 197, 216, 217
Acute myeloid leukemia (AML), 22, 35, 52, 157, 161, 166, 167, 171, 179, 203, 204, 220, 432, 1039, 1052, 1130, 1131, 1162, 1186, 1253, 1263, 1264
bone marrow biopsy, 380
chemotherapy for, 382–383
chest radiograph, 381
in children, 359, 370
chemotherapy, 365
classification, 361
clinical presentation, 360–361
core-binding factor, 362
cytogenetic and molecular features, 361–363
diagnosis, 361, 363
epidemiology, 359–360
immunophenotype analysis, 363
molecular abnormalities, 362

monitoring treatment response, 364–365
pathogenesis, 359
prognostic cytogenetic alterations, 363
relapsed and refractory, 369–370
remission induction, 364
treatment, 364
classification, 205
clinical features, 375–380
CNS leukemia, 395
cytarabine, 383
cytogenetics, 282–283
daunorubicin, 383, 384
developing, 395–396
diagnosis, 377, 380, 381
elderly patients, 393–395
European Leukemia Net Risk Stratification, 376
FAB classification, 376
FLT3 mutation in, 247, 289
gene alterations, 383
with gene mutation, 211–212
high-dose cytarabine, 378, 386
hypocellular (hypoplastic), 199
incidence, 179, 180
induction chemotherapy, 381–386
infection prevention methods, 381
leukemic clone in, 391
with maturation (M2), 214
megakaryocytes, 377
minimally differentiated, 213, 214, 240
mixed-phenotype acute leukemia, 395
myelodysplastic syndrome, 212, 387
not otherwise specified, 213
NPM1 (nucleophosmin) (5q31) mutation, 289
patient management, 375
physical examination, 380
poor prognostic factor, 380
postremission therapy, 382, 388–390
potassium wasting, 379
preleukemic phase, 387
prophylactic emergency treatment, 380
prostatic infiltration, 379
pulmonary dysfunction, 379
with recurrent cytogenetic abnormality, 206
refractory and relapsed, 391–393
relative risk for, 180
serum lactate dehydrogenase levels, 377
standard remission induction regimen, 386
with t(6;9)(p23;q34);DEK-NUP214, 210
testicular relapse, 379
t(8;16)(p11.2,p13.3)/*KAT6A*(*MOZ/MYST3*)–*CREBBP*(*CBP*), 287
with t(9;11)(p22-q23); MLLT3-MLL, 209
with t(1;22)(p13;q13); RBM15-MKL1, 210–211
triple-lumen Hickman catheter, 382
with t(8;21)(q22;q22);RUNX1-RUNX1T1, 206–207
WHO classification, 376
Acute Myeloid Leukemia Berlin–Frankfurt–Muenster (AML-BFM), 1254
Acute myelomonocytic leukemia, 214–216
Acute panmyelosis, 197
Acute panmyelosis with myelofibrosis (APMF), 219, 227
Acute progranulocytic leukemia (APL), 375
Acute promyelocytic leukemia (APL), 162, 163, 197, 361, 367, 368, 1159, 1263
and pregnancy, 440
arsenic trioxide, 410
bleeding diathesis, 443
in children, 440
clinical application, 427–429
clinical features, 430–432
coagulopathy and early death in, 441
coagulopathy associated with, 440–442
cytogenetic and molecular abnormality, t(15;17)(q24;q21)/*PML–RARA*, 283–284
death rate and bleeding, 441
diagnosis, 283
diagnostic hallmark of, 430
early death rate, 443, 444
FAB classification, 409
hematopoietic stem cell transplantation, 440
hemorrhagic death in, 443
high-risk patients, 432
immunophenotype of, 431
induction therapy, 433
leukemia-initiating cell, 411
leukemic cells in, 431
low-risk patients, 432
molecular biology, 409–411, 428
molecular genetic diagnosis, 427
MRD monitoring in, 429
normal and translocated chromosomes, 410
pathophysiology of coagulopathy, 441–442
postremission therapy in, 438–440
promyelocytes, 424
RARα, 411–413
relapsed APL, 440
retinoic acid receptor-alpha fusion genes, 419
treatment, 433, 439, 444
with t(15;17)/q22;q12, PML–RARA, 207–208
Acute tumor lysis syndrome, 344
Acute undifferentiated leukemia (AUL), 221
Acyclovir, 1081, 1089, 1091
Acyclovir-resistant HSV infection, 1082
Adenosine deaminase (ADA), 121, 141
Adenosine triphosphate (ATP), 1144
Adenoviruses, 1093
infection patterns, 1093
prevention, 1094
treatment, 1094
Adrenocorticotropic hormone, 695
Adult leukemia, 157
Adult T-cell leukemia virus (ATLV), 714
Adult T-cell leukemia/lymphoma (ATL/ATLL), 713, 730, 800–802, 835, 875
Affymax, 706
African Burkitt's lymphoma, 1090
African lymphoma, 768
Aggregation of exposures, 762
Aggressive NK cell leukaemia (ANKL), cytogenetic analysis, 875
Agranular blast, 224
AIDS *See* Acquired immunodeficiency syndrome (AIDS)
AIDS-associated lymphomas, treatment, 1008, 1009
AIDS–Burkitt's lymphomas, 1005
AIDS–non-Hodgkin's lymphomas, 1004, 1005, 1007, 1015
AILD *See* Angioimmunoblastic lymphoma with dysproteinemia (AILD)
Air quality, 1052
Akathisia, 1294
AL amyloidosis, 604
ALCL *See* Anaplastic large-cell lymphomas (ALCL)
Alcohol-induced pain, Hodgkin lymphoma, 908
Alemtuzumab, 147, 839
Alkaline phosphatase anti-alkaline phosphatase (APAAP) technique, 202

Alkylating agent, 121, 467, 519
  related leukemia, 923
  Hodgkin lymphoma, 693
ALL *See* Acute lymphocytic leukemia
All trans retinoic acid (ATRA), 1264
Allele level, 1186
Allele specific PCR (AS-PCR), 727
Allele-specific oligonucleotide PCR (ASO-PCR), 129
Alloantibody, 1040
Allogeneic bone marrow transplantation, 390, 1083
Allogeneic donor selection, 1182
Allogeneic HCT, 578
Allogeneic HSCT, 1182
Allogeneic stem cell transplantation (Allo-SCT), 62, 348, 563
Allogeneic transplantation, HLA-matched related and unrelated donors, 1186–1187
Alloimmunization, 1148, 1155, 1156, 1161, 1164
Allotransplantation, 4, 1256
All-trans retinoic acid (ATRA), 207, 240, 283, 409, 441
  binding pocket, 413
  bleeding diathesis, 443
  clinical results with, 434–435
  on coagulation parameters, 442–443
  exposure interval, 422
  high-sensitivity biological activities, 424
  induction therapy, 433
  liposomal ATRA, 435
  molecular mechanisms of, 425–427
  pharmacological concentration, 416
  pharmacology of, 433–434
  post-ATRA interval, 422
  postremission treatment, 434
  regulation, 412
  therapy, 420
Alpha heavy chain disease, 785
Alpha-2 antiplasmin, 442
Amantadine, 1100
Ambiguous lineage, acute leukemia of, 221
American Society for Apheresis, 1131
American Society of Clinical Oncology (ASCO), 1154, 1248, 1252
American Thoracic Society (ATS), 1067
AML *See* Acute myeloid leukemia (AML)
Amyloid, 651
  cardiomyopathy, 661
  immunoglobulin light chains, 652
  staging system, 659
  types, 655, 656
Amyloidosis, 651
  algorithm, 666
  antibodies, 666
  biomarkers, 659
  biopsy proof, 655
  bortezomib-based chemotherapy, 665–666
  chemotherapy, 661–662
  diagnosis, 653–654
  and heart, 657–658
  immunomodulatory drugs, 664–665
  localized and systemic, 656
  Mayo Clinic, 656, 661
  melphalan-based therapy, 661
  MRI, 658, 659
  periorbital purpura, 653
  renal, 659, 660
  stem cell transplantation, 662–664
  supportive care treatment, 660–661
  symptoms and signs, 652–653
  Systemic Forms of, 657
Anaplastic large cell lymphoma (ALCL), 730, 834, 835, 881, 965, 991
  ALK-, 808–809
  ALK+, 807–808
  breast implant-associated, 809
  clinical presentation, 990
  Cooperative Group trials, 992
  molecular biology, 989
  outcome, 991
  pathology, 989
  primary cutaneous, 990
  treatment, 990–993
Anaplastic lymphoma kinase (ALK), 807, 834
  ALK- ALCL, 808
  ALK+ ALCL, 807
  ALK+ large B-cell lymphoma, 794–795
Anemia, 80, 84, 496, 1250
Angioimmunoblastic lymphadenopathy, 1010
Angioimmunoblastic lymphoma with dysproteinemia (AILD), 834
Angioimmunoblastic T-cell lymphoma (AITL), 730, 805, 806, 880, 967
Angiotropic lymphoma. *See* Intravascular large B-cell lymphoma (IVLBCL)
Animalcules, 677
Annexin A1 (ANXA1), 138
Annexin-VIII, 442
Anthracycline, 321, 345
Anthracycline chemotherapy, 430, 432, 434–437, 444
Antibiotic-induced coagulopathy
  immune thrombocytopenia, 1275
  vitamin K-dependent clotting factor, 1274
Antibody, bi-specific, 255. *See also specific types of antibody*
Antibody-dependent cellular cytotoxicity (ADCC), 123, 1247
Anti-CD20 antibody, 521, 962
Anticipation, 135, 1294
Anti-CMV antiviral therapy, 1085
Anticoagulant–preservative solutions, 1144, 1145
Antidepressants, 1296
  side effects, 1297
  titration protocol, 1296
Antiemetics, 1043
Antifungal drug, 1071, 1074
Antifungal prophylaxis, 1054, 1055
Antigen, 237, 1186
  associated with prognostic gene expression, 247–249
  B-lineage-associated, 243
  co-expression, myeloid, 310
  for leukemia fusion gene, 241–242
Antigen–antibody interaction, 1146
Antigen-dependent cell-mediated cytotoxicity (ADCC), 127
Anti-HLA alloimmunization, 1165
Anti-HLA antigen-directed antibodies, 1161
Anti-IL-6 receptor (IL-6R) antibody, 1186
Anti-inflammatory cytokines, 1294
Antileukemic therapy, 1131
Anti-leukocyte antibodies, 1148
Antimicrobial prophylaxis, 1047, 1053, 1069, 1070
Antineoplastic chemotherapy, 1064
Antineoplastic therapy-associated neutropenia, 1063
Antiplatelet therapy, 1268, 1269
Antithymocyte globulin (ATG), 1181, 1187, 1216
Antiviral capsid antibody (VCA), 1090
Antiviral prophylaxis, 1055
  CMV, 1055
  EBV, 1056
  HBV and HCV, 1056

HSV, 1055, 1056
mycobacteria, 1057
Pneumocystis jirovecii, 1057
respiratory viruses, 1056, 1057
toxoplasmosis, 1057
VZV, 1056
Anxiety
adjustment disorder, 1293
anticipatory anxiety and nausea, 1294
defined, 1291, 1292
due to general medical condition, 1294
GAD, 1293
generalized disorder-7, 1292
maladaptive cancer cognitions, 1299
medical conditions and medications associated with, 1294
panic disorder with/without agoraphobia, 1293
phenotype components, 1291
prevalence, 1292
psychopharmacological treatments, 1296
risk factors, 1292
screening and measuring, 1292
social anxiety disorder, 1293
specific phobia, 1293
substance-induced, 1294
Apheresis, 1040, 1129, 1134
complications of, 1130
therapeutic concept, 1129
APL. *See* Acute promyelocytic leukemia (APL)
Aplastic anemia, 309
APMF. *See* Acute panmyelosis with myelofibrosis (APMF)
Apolipoprotein (APOE), 1303
Apoptosis, 72, 73, 107
Army General Classification Test, 759
Array technology, 1195
Array-comparative genomic hybridization (aCGH), 282, 729
Arsenic trioxide (ATO), 368, 410, 428, 435
APL blast count, 427
cell biological activity, 423
clinical results with, 436–437
concentrations, 423
GSH system, 423
high-sensitivity biological activities, 424
molecular mechanisms of, 425
PML-RARα degradation, 423
salvage therapy, 429
sensitivity, 421
toxicity of, 437–438
Arterial and microvascular thrombosis, JAK2 inhibitors treatment, 1269
Asparaginase, 345
Aspergillosis, 1072
Aspergillus sp., 1042, 1073
Aspirin, 1268
Ataxia telangiectasia and RAD3-related (ATR) nuclear protein kinase signaling, 35
Ataxia telangiectasia-mutated (*ATM*) gene, 101, 119
ATO. *See* Arsenic trioxide (ATO)
ATP. *See* Adenosine triphosphate (ATP)
ATRA. *See* All-trans retinoic acid (ATRA)
Atypical chronic myeloid leukemia (aCML), 22
BCR-ABL1 negative, 229
bone marrow cytology, 23
genetics, 23
histology, 23
peripheral blood count and cytology, 22–23
Atypical CLL, 80, 83
Atypical localization of immature progenitor cells (ALIP), 224
Auer rod, 204, 431
Autoantibody activity, WM, 622
Autoimmune cytopenias, 80
Autoimmune diseases (AD), 469, 1018, 1020
Autoimmune hemolytic anemia (AIHA), 118, 129, 1143
Autoimmune thrombocytopenia (AIT), 118, 129
Autologous and allogeneic peripheral blood stem cell, 1184
Autologous bone marrow transplantation (ABMT), 390, 393, 910, 983
Autologous frozen platelets, 1157
Autologous hematopoietic cell transplantation (autoHSCT), 470
Autologous stem cell transplantation (ASCT), 440, 551, 559, 639, 1185
Autologous transplantation
allogeneic *vs.*, 1182
autoimmune disorders, 1185
growth factors
chemotherapy, 1255
hematologic cells production, 1255
hematologic malignancies, 1255
malignancies, 1185
Azacitidine, 394, 503
Azole, 1073, 1074

B

B cells, 999
B lymphocyte, 703
Bacteremia, 1041
Bacterial contamination, of platelets, 1166
Bacterial infections, hematological malignancies, 1063
antimicrobial prophylaxis, 1069, 1070
diagnosis, 1066
epidemiology, 1064
risk factors, 1064, 1065
treatment, 1067, 1068
Baculoviral IAP repeat containing 3gene (*BIRC3)*, 779
Basic helix–loop–helix (bHLH) transcription factor, 165
B-cell
in follicles, 773
Ig light chain, 777
immunoglobulin synthesis by, 773
lymphocytosis, monoclonal, 79
mitogen, polyclonal, 99
prolymphocytic leukemia, 141
receptor signaling, 107
B-cell disorder, chronic, 117
B-cell lymphoma, 723, 783, 798, 828, 1092
ALK-positive large, cytogenetic analysis, 870
classical Hodgkin lymphoma, 799
high-grade (*see* High-grade B-cell lymphomas)
B-cell lymphoma 2 modifying factor gene (*BMF*), 108
B-cell lymphoproliferative disorder, 141
B-cell non-Hodgkin lymphoma (B-NHL), 983, 1010, 1185
B-cell precursor (BCP) ALL, cytogenetics, 297
chromosome numbers, 290–293
gene rearrangements associated with favorable prognosis, 293
structural abnormalities, 293
B-cell prolymphocytic leukemia (B-PLL), 82, 117, 856
B-cell receptor (BCR), 245
inhibitors of, 124
pathway, 5
B-cell type non-Hodgkin's lymphoma, 829
BCL11B gene, 288
BCL2 gene, 791
Bcl-2 protein, 782

BCL2/6 translocations, 798
BCL6 genes, 793
BCR-ABL gene, 3, 165
BCR-ABL1, 30–32, 38, 40, 49, 55
  CML BP cells overexpress, 34
  expression, 35
  fusion gene, 50
    detection, 49
    in interphase cells, 55
  oncogene, 29, 30
  oncoprotein, 29
  protein kinase
    autoregulation, 30–31
    domain mutation, 38, 40
    signaling pathways stemming from, 31–32
    structure, 30
BCR-ABL1-positive ALL, 312
Behavioral therapies, 1299
Behvioral activation (BA), in depressed patients, 1299
Bence-Jones proteins, 651
Bendamustine, 121, 147, 787
Bendamustine and rituximab (BR), 122
Benzodiazepines, 1296–1298, 1301
Berlin-Frankfurt Muenster (BFM) study, 370, 980
β-D-glucan testing, 1071
BGB-3111, 127
Bilineal leukemia (BLL), 238
Bimolane, 467
Bing-Neel syndrome, 625
Biomarker
  amyloidosis, 659
  Hodgkin lymphoma, 907
Biopsy, amyloidosis, 655
Bipolar disorder, 1295
Bi-specific antibody, 255
Bi-specific T-cell engagers (BiTE), 255
Bisphosphonate-associated osteonecrosis of the jaw (BRONJ), 598, 599
BL. *See* Burkitt lymphoma (BL)
Blast cell, 225, 343, 1130
  characteristics, 224
  immunophenotype, 267
Blastic crisis of chronic myeloid leukemia (CML-BC), 1130, 1131
Blastic plasmacytoid dendritic cell neoplasm (BPDCN), 220
Blastoid MCLs, 788
Bleeding diathesis, 443
Blinatumomab, 255, 354
B-lineage ALL, 341
  BCR/ABL1-like/Ph-like, 251–252
  MFC-MRD detection in, 265
B-lineage-associated antigen, 243
Blood abnormality, Waldenstrom's macroglobulinemia, 625
Blood and Marrow Transplant Clinical Trials Network (BMT-CTN), 578
Blood product transfusions, 1039
Blood–injection–injury phobia, 1293
Bloom's syndrome, 1026
B-lymphoblast, 238
B-lymphoblastic leukemia/lymphoma (B-ALL/LBL), 777, 987
  not otherwise specified, 776–777
  with recurrent genetic abnormalities, 777
B-lymphocyte differentiation, 977
B-lymphoid differentiation, 827
B-lymphoid neoplasms, WHO classification, 775–776
BMT. *See* Bone marrow transplants (BMT)
Body cavity-based lymphoma *See* Primary effusion lymphoma (PEL)
Body surface area (BSA) ratio, 1168
Bone marrow, 85, 1183
  aspiration, 226, 361
  biopsy, 361
  cytology, CML, 20
  fibrosis, 227
  film, 21
  histology, 224–225
  hypoplasia, 386
  involvement, 789
  trephine biopsy, 21, 22
Bone marrow plasma cells (BMPCs), 531
Bone marrow transplants (BMT), 197, 1080
  clinical features, 1017
  epidemiology/demographics, 1016, 1017
  histopathology, 1017
  iatrogenic immune suppression, 999
  pathogenesis, 1017
Bone marrow *vs.* peripheral blood, 1183
Bone marrow/progenitor cell transplantation, 1143
Borrelia burgdorferi, 784, 1011, 1012
Bortezomib, 564, 565
  PCL, 641
  chemotherapy, amyloidosis, 665–666
Bortezomib-cyclophosphamide-dexamethasone (VCD), 556–557
Bortezomib-dexamethasone (VD), 558
Bortezomib-doxorubicin-dexamethasone (PAD), 557
Bortezomib-lenalidomide-dexamethasone (VRD), 556
Bortezomib-thalidomide-dexamethasone (VTD), 556
Bosutinib, for CML, 59
BPDCN. *See* Blastic plasmacytoid dendritic cell neoplasm (BPDCN)
B-PLL. *See* B-cell prolymphocytic leukaemia (B-PLL)
B-progenitor acute lymphoblastic leukemia (B-ALL), 169
  CD20 antibody in, 252
  CD22 antibody in, 254
  cytogenetic abnormalities in childhood, 311
  immunophenotype, 309–310
  white blood cell count, 351
B-prolymphocytic leukemia (B-PLL), 93
Bradycardia, 664
BRAF inhibitors, 145
Breast cancer, 675, 676, 944
Breast implant-associated ALCL, 809
Brentuximab vedotin (BV), 840, 916, 918, 919
  CAR T cells, 841
  radioimmunotherapy, 841, 842
Brill–Symmers disease, 700
Brincidofovir, 1086
British Amyloidosis Center, 663
Bruton's tyrosine kinase (BTK), 5, 111, 125, 126, 246, 618, 619, 728, 1027, 1274
BTK. *See* Bruton's tyrosine kinase (BTK)
Buffy coat (BC), of WBC, 1154
Bupropion, 1297
Burkitt lymphoma (BL), 200, 711, 712, 768, 769, 796–798, 831, 833, 964, 999–1001, 1005, 1027
  advanced-stage, 982
  chemotherapy, 964
  clinical features, 953
  clinical presentation, 981
  Cooperative Group, 984
  cytogenetic analysis, 871, 872
  diagnosis, 953
  EBV, 709–713, 1002
  with 11q aberration, 798
  genetic analysis, 729–730

HIV patients, 1007
laboratory analysis, 958
molecular biology, 981
paraffin-embedded tissue, 872
pathology, 980
relapsed, 964
treatment, 981–984, 992
Burkitt lymphoma/leukemia (mature B-ALL), 201
Burnout and compassion fatigue, 1303
Bursectomy, 702, 703
Busulfan, 53
BV. *See* Brentuximab vedotin (BV)

C
Calcineurin inhibitor (CNI), 1276
C*ampylobacter jejuni*, 623
Campylobacter pyloris, 717
Cancer
ancient oncology, 675–677
lymphatic system and, 678–680 (*see also* Lymphoma)
microscopy/examination of tissues, 677–678
modern times, 675
neurocognitive effects of, 1303
radiation treatment, 690–693
soft tissue, 675
Cancer and Leukemia Group B (CALGB), 910
Cancer Chemotherapy National Service Center, 699
Cancer procoagulant (CP) protein, 442
Cancer support groups, 1300
Candida infections, 1070
antifungal prophylaxis, 1071
clinical presentation, 1070
diagnosis, 1071
epidemiology, 1070
risk factors, 1070
treatment, 1071
Candida spp., 1068, 1070
Candidate gene study, 739, 740
germline genomic, 735, 739
cytokine genes, 739
DNA and chromosomal integrity, 740
FCGR2A/FCGR3A, 740
GWAS, 740
pharmacogenetics and metabolis, 740
Carbapenem-resistant enterobacteriaceae (CRE), 1064
Cardiac amyloidosis, 658, 660
African-American male, 657
MRI, 658
presentation, 658
sudden death, 657
symptoms, 659
ventricular dysfunction, 656
wild-type TTR, 656, 658
Cardiac arrhythmia, 660
Cardiac late effects, 321
Cardiac transplantation, 660, 661
Cardiomyopathy, infiltrative, 652, 654
Cardiovascular complications, Hodgkin lymphoma, 921–922
Care managers, 1304
Carfilzomib, 630
Carfilzomib-lenalidomide-dexamethasone (KRD), 557–558
Carpal tunnel syndrome, 656
CAR-T cell, 129
therapy, 1185–1186
Casteman's disease, 1092
Catalogue of somatic mutations in cancer (COSMIC), 724
Catharanthus roseus, 696
CCAAT-enhancer-binding protein alpha (CEBPA), 168–169
CD13, 436
CD20 antibody, 123, 252, 253
CD22 antibody, in B-ALL, 254
CD25 antibody, 254–255
CD45 antibody, 1181
CD52 antibody, 123, 124, 254
CD20-directed antibody therapy, Waldenstrom's macroglobulinemia, 628
CD34 expression, 440
CD38 expression, 120
CD5–/CD10–lymphoma, 830, 831
AILD, 834
ALCL, 834, 835
alemtuzumab, 839
ATLL, 835
BL, 833
CD5–/CD10+, 831
CTCLs, 835, 836
dacetuzumab, 839
DLBCL, 831
epratuzumab, 839
FL, 832
HL, 836–838
immune checkpoint blockade, 840
immunotherapy, 838
ipilimumab, 840
lucatumumab, 839
MALT lymphoma, 830
mogalizumab, 839
NK/T, 836
obinutuzumab, 838, 839
ofatumumab, 838
PD-1 inhibitors, 840
PTCL, 833
rituximab, 838
T-cell non-Hodgkin's lymphomas, 833
CD33 monoclonal antibody, 254, 475
CD19-targeted therapy, 256
CD4+ T cells, 1131, 1132
CEBPA gene mutation, 168, 290
CEBPB transcription, 421
Cell cycle, 107
Cell of origin (COO), 689, 725
Cell surface marker, 310
Cellular anti-infection therapy, 1169
Cellular pathology, 699
Cellular plasticity, 107
Cellular retinoic acid binding protein (CRABP), 425
Center for International Blood and Marrow Transplant Research (CIBMTR), 574, 643, 993, 1083, 1084, 1187, 1199, 1212
Centerin. *See* Germinal center B-cell expressed transcript-1 (GCET)
Central line associated bloodstream infections (CLABSIs), 1068
Central nervous system (CNS), 979, 999
DLBCL, 868
leukemia, 221, 315, 317, 320, 347, 395
risk, 953
therapy, 320
Waldenstrom's macroglobulinemia, 625
Central venous catheters (CVCs), 1064, 1217
Centroblasts, 781
Centrocytes, 781
Cerebrospinal fluid (CSF), 339, 381
Chemoimmunotherapy, 5

Chemokine receptor CXCR4, 1254
Chemotherapeutic agents, 121, 1275
Chemotherapy, 693–697, 910, 919, 939–941, 944, 1275
 for AML, 382
 Burkitt lymphoma, 964
 CHOP, 981
 CNS prophylaxis, 953
 combined modality, 956
 gonadotoxicity, 925
 high-grade lymphoma, 946
 Hodgkin lymphoma, 912, 913, 942
  alkylating agents, 693–695
  combined drug therapy, 696–697
  glucocorticoids, 695–696
  relapse, 919
  vinca alkaloids, 696
 indolent NHL, 960
 intermediate-grade lymphomas, 945–946
 light chain amyloidosis, 661–662
 lymphoma
  combined-modality therapy, 940, 941
  dose–response, 939–940
  low-grade, 945
  risk-/response-adapted therapy, 941
  surveillance, 944
 multi-agent, 942
 NHL, 945
 partial response, 944
 principles, 960
 Stanford V, 943
Childhood lymphoblastic leukemia, 157
Childhood non-Hodgkin lymphoma (NHL), 975
 classifications, 976–977
 clinical presentation, 978
 diagnosis, 978–979
 epidemiology, 975
 etiology, 975–976
 molecular biology, 978
 pathology, 977–978
 risk stratification, 979
 staging, 979–980
 treatment approaches, 980
Childhood sexual abuse, 1292
Chimeric antigen receptor (CARs), 1186
Chimeric antigen receptor-engineered T-cells (CAR-T-cells), 6, 252, 255, 256, 354, 840, 841
Chimerization, 708
Chlamydophila psittaci (CP), 784, 1011
Chlorambucil, 626
2-Chlorodeoxyadenosine (2-CdA), 141
Chloroma, 52, 365
Chorioretinitis, 1082
Chromatin modifiers, 107, 169–171
Chromatin immunoprecipitation (ChIP) analysis, 169
Chromosomal abnormalities, 35, 162, 222
Chromosomal band, 13q14 deletion, 100–101
Chromosomal inversion, 163
Chromosomal translocation, 774
Chromosome
 chromosome 5 and 7, recurrent abnormality, 287
 copy number aberrations, 102
 rearrangements, 103
 translocation, 162, 298
Chromothripsis, 103
Chronic anemia, 1140, 1142
Chronic B-cell
 disorder, 117
 lymphoproliferative disorder, 141
Chronic disseminated candidiasis, 1070
Chronic eosinophilic leukemia (CEL), 24
 bone marrow cytology, 25
 flow cytometric immunophenotyping, 25
 genetics, 25
 histology, 25
 peripheral blood count and cytology, 24–25
Chronic graft-versus-host disease (cGVHD), 1132
 classification systems, 1230
 HLA disparity, 1230
 morbidity and mortality, 1228
Chronic hematologic disorders, 1157
Chronic Kidney Disease Epidemiology Collaboration (CKD-EPI) equation, 602
Chronic lymphocytic leukemia (CLL), 4–6, 69, 71, 79, 81, 83, 85, 99, 120, 253, 726, 777–779, 830, 905, 1132, 1141, 1255
 adoptive cellular therapy, 129
 antibodies, 123–124
 bendamustine for, 121
 biology, genetic associations, 72
 burden of, 117
 cell of origin, 81
 chemoimmunotherapy, 121–122
 chromosomal abnormalities, 100–103
 clinical features, 117–118
 clinical presentation, 953
 clonal evolution, 107–108
 copy number changes in, 100
 cytogenetic analysis, 853
 definitions, 79–80
 deoxycoformycin for, 121
 diagnosis, 117
 differential diagnosis, 91–93
 environmental-lifestyle risk factors, 70
 epidemiology, 69–70
 epidemiology and clinical aspects, 80–81
 epigenetic abnormalities, 108
 factors
  identify patients to treatment, 120
  impact overall survival, 120
  impact response to therapy, 120
 familial clustering of, 70–71
 fludarabine for, 121
 genetic predisposition to, 108–109
 genetic studies, 119
 genetic susceptibility, 71–72
 genome-wide association study, 72, 735
 genomic abnormalities in clinical practice, 110
 genomic complexity, 103
 germline variants and tumor, 74
 ibrutinib for, 125
 immune dysfunction as risk factor, 70
 immune-mediated cytopenia in, 129
 immunophenotype, 85–88
 incidence rates, 69
 laboratory parameters, 118–119
 linkage analysis, 71
 minimal residual disease testing in, 91
 monoclonal B-cell lymphocytosis as precursor condition, 74
 morphology
  bone marrow, 83
  lymph node, 85
  peripheral blood, 81
  spleen, 85

mutated, 81
mutational landscape, 104–107
pathogenesis, 81, 100
pentostatin for, 121
prognostic scoring system, 856
reciprocal translocations, 854, 855
risk variants, mechanism of effect, 74
SNP-A studies, 856
somatic hypermutation of immunoglobulin heavy-chain variable gene, 119
staging, 118
stereotyped B-cell receptor, 119–120
targeted therapy, 94
targeting minimal residual disease in, 129
transformation, 88–91, 130
treatment, 100, 120, 121
unmutated, 81
ZAP-70 and CD38 expression, 120
Chronic lymphoproliferative disorder of NK cells (CLPD-NK), 874
Chronic myeloid leukemia (CML), 3, 4, 19, 29, 37, 38, 49–51, 53–58, 61, 62, 165, 245, 312, 432, 1042, 1084, 1167, 1216, 1263
accelerated phase, 51–52
advanced-phase disease, 62
arrest of differentiation, 34–35
blast phase, 52
bone marrow cytology, 20–21
bosutinib for, 59
BP cells overexpress BCR-ABL1, 34
chromosomal abnormalities in, 35
chronic phase, 54
clinical presentation, 50
dasatinib (sprycel) for, 58
diagnosis, 13–14
diagnosis and differential diagnosis, 49–50
epidemiology, 9
etiology, 9–10
evaluation, 50
genomic instability and DNA repair, 35
health care and daily practice, 12–14
histology, 21–22
imatinib for, 54–55
dosage, 55
evaluation of response, 57–58
managing the patient, 58
resistance, 56–57
side effects, 55
stopping, responding patient, 61–62
incidence, 10–11
nilotinib (tasigna) for, 58–59
omacetaxine (homoharringtonine) for, 59
peripheral blood count and cytology, 19–20
Ph/BCR-ABL-positive, 9, 10, 14
ponatinib for, 59
prevalence, 11–12
progenitor, 33–34
prognostic scores, 52, 53
progression to advanced-phase, 32–33
quiescence and persistence, 36–37
secondary malignancy, 12
stem cell, 36–38
and survival rates, 11
treatment, 53
hydroxyurea (hydroxycarbamide)/busulfan (myleran), 53–54
immediate management of newly diagnosed patients, 53
interferon-alpha, 54
tumor-suppressor gene inactivation, 35–36
Chronic myelomonocytic leukemia (CMML), 23, 228, 229
bone marrow cytology, 24
flow cytometric immunophenotyping, 24
histology, 24
peripheral blood count and cytology, 23
Chronic neutrophilic leukemia, 25–26
CIBMTR analysis, 1199
CID *See* Congenital immunodeficiency diseases (CID)
Cidofovir, 1087
Cigarette smoking, 182, 187
Circulatory congestion, 1166
Citrate anticoagulant, 1155
Citrate-phosphated dextrose (CPD), 1130
Cladribine, 141, 142, 144
Clasmatocyte, 701
Classical Hodgkin lymphoma (CHL), 729, 799, 811, 812, 836, 870, 903
clinical outcome, 904
cytogenetic analysis, 873, 882
diagnosis of, 774
GWAS, 735–738
HIV, 905, 906
Classical Hodgkin Lymphoma-Richter Transformation (CHL-RT), 90
Clathrin (*CLTC*), 808
Claustrophobia, 1294
CLL. *See* Chronic lymphocytic leukemia (CLL)
Clofarabine, 393
Clonal Cytopenia of Undetermined Significance (CCUS), 494
Clonal hematopoiesis of indeterminate potential (CHIP), 363, 486
Clonal heterogeneity, 867
Clonal myeloproliferative neoplasia, 29
Clone nature, WM, 617
CML. *See* Chronic myeloid leukemia (CML)
CMML. *See* Chronic myelomonocytic leukemia (CMML)
CMV. *See* Cytomegalovirus (CMV)
CNA. *See* Copy number alteration (CNA)
Cognitive therapy, 1298, 1299
Cohesin, 171
Cohort study, 762
familial predisposition, 732
Hodgkin lymphoma, 760
Coiled-coil region, 414
Cold agglutinin activity, WM, 623
Collaborative care, 1304
Colony-stimulating factor (CSF), 203, 361, 1058
Colony-stimulating factor–granulocyte (G-CSF), 344
Combination chemotherapy, 519
Combination therapy, Waldenstrom's macroglobulinemia, 628
Combined anticoagulation, 1269
Combined drug therapy, Hodgkin lymphoma, 696
Combined-modality therapy (CMT), 911–914, 917, 922, 923
advantages, 941
local–regional therapy, 940
operator dependence, 940
patterns of failure, 941
Communication skill training, 1303, 1304
Comorbidity, 540
Comparative genomic hybridization (CGH), 99
Complementarity-determining region (CDR), 120
Complementarity-determining region 3 (CDR3), 602
Complementary DNA (cDNA), 428
Complement-dependent cytotoxicity (CDC), 123
Complete cytogenetic response (CCyR), 51
Complete molecular response (CMR), 56
Complete remission (CR), 409, 1180
Complex karyotype, 287–288

Comprehensive geriatric assessment (CGA), 541
Computed tomography (CT)
  lymphoma, 688
  Waldenstrom's macroglobulinemia, 626
Conditioning regimen intensity, 1216
Confusion, 1300
Congenital immunodeficiency diseases (CID), 976, 1021–1025
  clinical features, 1021
  EBV, 1027
  histopathology, 1021
  pathogenesis, 1026
  treatment, 1027, 1028
Congo red, 651, 652, 655, 658
Consolidation therapy, 388
  ALL, 319
  Hodgkin lymphoma, 916, 917
Conventional cytogenetics, 290
Conventional procedure (cRT-PCR), 428, 429
Conventional respiratory viruses (CRVs), 1098
Conventional salvage therapy, 965
Coolidge tube, 691
Cooperative Group trial
  ALCL, 992
  Burkitt lymphoma, 984
  DLBCL, 989
  lymphoblastic lymphoma, 987
Copy number alteration (CNA), 99
Cord blood cells, 1184
Cord blood monocytes, 1183
Cord blood transplantation (CBT), 1187
Core-binding factor (CBF)
  AML, 362
  gene, 209
  leukemia
    11q23/*KMT2A* (*MLL*, *MLL1*, *ALL1*, *TRX*, *HTRX1*), 285–286
    3q26.2/*MECOM*(*EVI1*, *MDS1*) and 3q21.3/*GATA2*/*RPN1* rearrangements, 285
    inv(16)(p13.1q22) and t(16,16)(p13.1,q22)/*CBFB-MYH11*(*SMMHC*), 285
    RUNX1(*AML1*,*CBFA2*) (21q22) rearrangements, 285
    t(1,22)(p13,q13)/*RBM15*(*OTT*)–*MKL1*(*MAL*), 287
    t(10,11)(p12,q14)/*PICALM*(*CALM*)–*MLLT10*(*AF10*), 286
    t(6,9)(p23,q34)/*DEK–NUP214*(*CAN*), 287
    t(8,21)(q22,q22)/*RUNX1*(*AML1*,*CBFA2*)-*RUNX1T1*(*ETO*,*MTG8*), 285
    t(3,5)(q25,q35)/*NPM1–MLF1*, 287
    t(X,6)(p11.2,q23.3)/*GATA1-MYB*, 287
  rearrangement, 164
    BCR/ABL, 165
    CBFB/MYH11, 164–165
    E2A fusion, 165–166
    ETV6/RUNX1, 165
    RUNX1/CBFA2T1, 164
Coronary angiography, 657
Corticosteroids (dexamethasone), 519, 520, 546, 1219, 1294
Corynebacterium simplex, 696
Cotswold modification, 955
Cotswolds Committee, 907
Coulter counter, 705
Coxsackie A1, 1092
Coxsackievirus infection, 1092
CpG oligonucleotide, 99
CPX-351, 475
CRABP2, 426
Creatinine clearance (CrCl), 611
CREBBP, 170
Cross-reactive groups (CREGs), 1163
Cryoglobulinemia, 622, 1134
Cryptic chromosomal abnormality
  14q32/*BCL11B*, 288
  inv(16)(p13.3q24.3)/*CBFA2T3(MTG16/ETO2)–GLIS2*, 288
  NUP98 (11p15.5) gene, 288
  t(7,12)(q36.3,p13)/*MNX1–ETV6*, 288
CSF. *See* Colony-stimulating factors (CSF)
CSF3. *See* Granulocyte colony-stimulating factor (G-CSF)
CTCL. *See* Cutaneous T-cell lymphoma (CTCL)
CTLp frequencies, 1201
Current American Association of Blood Banks (AABB), 1154
Cutaneous lymphoma, 707–708
Cutaneous T-cell lymphoma (CTCL), 835, 836, 878, 879, 1131, 1132
C-X-C chemokine receptor type 4 (*CXCR4*), 728
  mutations, 780
  WHIM mutation, 619, 620
Cyclic adenosine monophosphate (cAMP) signaling, 423
Cyclin D1-negative MCL, 789
Cyclin E, 793
Cyclophosphamide, 121
Cyclophosphamide, doxorubicin, vincristine, and prednisone (CHOP) chemotherapy, 945, 981
Cyclosporin A, 387
Cyclosporine, 1013
CYP26A1, 426
Cysteine/histidine-rich (Cys3HisCys4) cluster, 413
Cytapheresis, 1130–1132
  ALL, 1131
  AML and CML, 1130, 1131
  SS, 1131, 1132
Cytarabine, 347, 384, 430, 433, 502
Cytochemical stain, 197, 202
Cytochrome P-450 (CYP450), 1008, 1297
Cytogenetic abnormality, in CLL, 99, 100, 102
Cytogenetic analysis, 488, 647
  acute leukemia, 281
  acute myeloid leukemia, 282
  AITL, 880
  ALCL, 881
  ALK-positive large B-cell lymphoma, 870
  ANKL, 875
  ATLL, 290, 875
  B-PLL, 290, 856, 857
  Burkitt lymphoma, 871, 872
  CHL, 873, 882–884
  CLL, 853–856
  CLPD-NK, 874
  CNS DLBL, 868–869
  DLBCL, 865, 872, 873
  EATL, 877
  EBV-positive DLBCL, 869
  ENK/TL-NT, 876
  follicular lymphoma, 860–862
  hairy cell leukemia, 136, 857
  Hodgkin lymphoma, 882
  HSTL, 877
  lymphoma, 853
  lymphoplasmacytic lymphoma, 857–858
  MALT, 858–860
  mantle cell lymphoma, 862–865
  mycosis fungoides, 878
  NLPHL, 884–885
  nodal MZL, 860
  PMBL, 869–870
  primary cutaneous DLBCL, 869

PTCL NOS, 879
Sézary syndrome, 878
SLL, 853
SMZL, 857
T-cell large granular lymphocytic leukaemia, 874
THRLBCL, 868
T-PLL, 873
Cytokine receptor-like factor 2 (CRLF2), 203, 313
Cytokine release syndrome (CRS), 256
Cytokines, 442
Cytomegalovirus (CMV), 1006, 1055, 1082, 1140, 1142, 1143, 1147, 1164, 1198, 1276
bone marrow transplant patients, 1085
diagnosis, 1084–1088
infections, 1166
infection and disease, 1219–1220
infection patterns, 1082–1084
positive donors, 1166
prophylaxix, 1084–1088
treatment, 1084–1088
Cytopenia, 1042
immune-mediated, 129
vs. morphologic dysplasia, 226
Cytoplasmic azurophilic granule, 204
Cytoplasmic bleb, 210
Cytoplasmic granules, 431
Cytoreductive therapy, 1080
Cytospin preparation, 206
Cytotoxic T lymphocyte precursor (CTLp), 1201
Cytotoxic T lymphocytes (CTLs), 1001

D
Dacetuzumab, 839
90Y–Daclizumab, 919
Danazol, 499, 500
Darbepoetin, 500
Dasatinib (Sprycel), 40, 58, 1274
Data Safety and Monitoring Committee, 1159
Daunorubicin (DNR), 345, 383, 384, 409, 433, 473
Daxx, 419, 421
Decitabine (DAC), 394, 503
Decreased platelet function, 1267
Deferasirox, 500
Delirium, 1300
clinical features, 1300
clinical risk factors for, 1301
course, 1300
management, 1301
mortality, 1301
risk factors, 1300
Denosumab, 599
Densitometry, 625
Deoxycoformycin, 121, 143
Depression
anxiety and suicide, 1296
collaborative care, 1304
defined, 1294
differential diagnosis, 1295
measuring and screening, 1295
medical workup, 1296
prevalence and impact, 1294, 1295
psychopharmacological treatments, 1296
risk factors, 1295
Dermal–epidermal junction, 1167
Dexamethasone, 345, 664
Dexmedetomidine, 1301
Dextroamphetamine, 1298
di(2-ethyl)phthalate (DEHP), 1145
Diet, 1052
Differentiation syndrome, 435
Diffuse large B-cell lymphoma (DLBCL), 109, 725, 768, 789, 790, 830–832, 963, 1003, 1007, 1185
clinical features, 953
clinical presentation, 988
clinical trials, 963
CNS, 868
Cooperative Group trials, 989
cytogenetic analysis, 865, 872, 873
EBV-positive, 869
GWAS, 738
histologic transformation, 955, 956
laboratory analysis, 957
molecular abnormality, 957
molecular biology, 988
NOS, 789–791
outcome, 989
pathology, 988
primary cutaneous, 869
treatment, 988–989
Diffuse large B-cell lymphoma-Richter transformation (DLBCL-RT), 89–91
Dimethyl sulfoxide (DMSO), 1156
Diploidy. *See specific types of diploidy*
Direct acting antivral (DAA), 1107
Direct antiglobulin test (DAT), 1221
Direct infection model, 1000
Directigen Flu A test, 1100
2,3-Diphosphoglycerate (2,3-DPG), 1144
Disability, 541
Disease transformation, 109
Disseminated intravascular coagulation (DIC), 207, 309, 361, 368, 1264
Distraction, 1299
Distress Thermometer, 1292
Diuretics, 652, 659, 660
DLBCL. *See* Diffuse large B-cell lymphoma (DLBCL)
DLBCL-RT. *See* Diffuse large B-cell lymphoma-Richter transformation (DLBCL-RT)
D-methionine, 1223
DNA binding domain (DBD), 411
DNA damage, 35, 107
DNA double-stranded breaks (DNA DSB), 119
DNA methyltransferase inhibitors, 503
DNA polymerase, 1082
DNA probe, 119, 281
DNA repair, genomic instability and, 35
DNA-based typing methods, 1198
Döhner model, 99, 111
Donor HLA incompatibility, clinical impact, 1198
Donor lymphocyte infusion (DLI), 1216
Donor mismatching
alleles *vs.* antigens, 1200
class I molecules, 1201
for Class II Residues, 1201
Dose–response phenomenon, 1163
Double-esterase reaction, 216
Double-hit lymphoma (DHL), 791, 798, 873
Down syndrome (DS), 210, 317, 359
myeloid proliferations related to, 220
Down syndrome-myeloid leukemia (DS-ML), 368, 369
DUSP22, 809

Dyserythropoiesis (DysE), 213, 224
Dysgranulopoiesis (DysG), 213, 222
Dysmegakaryopoiesis (DysM), 212, 222–224
Dysmonocytopoiesis, 224
Dysmorphic red cell, 226
Dysplasia
  morphologic, 212
  multilineage, 212
Dysplastic granulocyte, 223
Dysplastic megakaryocytes, 224
Dysplastic promyelocytes, 492
Dysproteinemic syndromes, 1271

E
E2A fusion, 165
Early B-cell factor (EBF), 837
Early T-cell precursor (ETP), 314
  ALL, 310, 314
  leukemia, 249
Early T-precursor acute lymphoblastic leukemia (ETP-ALL), 800
Eastern Cooperative Oncology Group (ECOG), 942, 945
EATL. *See* Enteropathy-associated T-cell lymphoma (EATL)
EBV. *See* Epstein–Barr virus (EBV)
EBV small encoded RNA (EBER), 795
EBV-associated lymphoproliferative disorders, 1091
EBV-encoded RNAs (EBERs), 1090
EBV-positive DLBCL, cytogenetic analysis, 869
Echinocandin, 1074
Echocardiography
  amyloidosis, 653
  Doppler studies on, 657
Electron microscope, 660, 678
Electrophoretic technique, 702
Eltrombopag (Promacta), 1251
EMP. *See* Extramedullary plasmacytoma (EMP)
Empiric therapy, 1042
Endemic BL (eBL), 729, 796
Endocrinopathy, 610
Endogenous virus, 1079
Endoscopic biopsy, 1080
Endoscopy, 655
Enhancer of zeste homologue 2 (EZH2), 170, 171
Enteropathy-associated T-cell lymphoma (EATL), 802, 803, 1020
  cytogenetic analysis, 877
Environmental exposure
  Hodgkin lymphoma, 762
  non-Hodgkin lymphoma, 769
Environmental precautions
  hospital environment, 1052
  hospital precautions, 1052
  household exposure, 1052
Enzyme immunoassay (EIA), 1106
Enzyme-linked immunoabsorbent assay (ELISA), 1095, 1164
  cross-matching technique, 1164
Eplets, 1164
Epratuzumab, 254, 839
Epstein–Barr virus (EBV), 689, 711, 729, 755, 759, 792, 833, 999–1003, 1056, 1166
  AIDS and, 765
  Burkitt lymphoma, 709–713, 768, 769
  chronic effect, 976
  diagnosis, 1090–1092
  genome, 975
  Hodgkin lymphoma, 760
  immunity, 1090
  lymphomas, 1000, 1003
    Burkitt's lymphoma, 1001, 1002
    DLBCL, 1003
    Hodgkin's lymphoma, 1002, 1003
  NHL, 951
  pathogenesis, 1090
  and Reed–Sternberg cell, 903
  significance, 689
  thyroid lymphomas, 1003
  treatment, 1090–1092
E-rosette receptor, 828
Erythrocytapheresis, 1133
Erythroid hyperplasia, 84, 472
Erythroid leukemia, 218
Erythroleukemia, 364
Erythromelalgia, 1266
Erythropoiesis stimulating agents (ESAs), 500, 501, 1144, 1250, 1255
Erythropoietin, 1144
  anemia, 1250
  ESA, 1250
  glycoprotein, 1250
  MDS, 1250
Erythropoietin receptor (EPOR), 203
Esophagitis, HSV, 1080
Essential thrombocythemia (ET), 1265
ETV6-RUNX1 fusion, 165, 312
European Bone and Marrow Transplant Registry, 1083
European Group for Blood and Marrow Transplantation (EBMT), 642, 643, 1085, 1214, 1227, 1249
European HOVON German Cooperative Group, 663
European LeukemiaNet (ELN), 12, 57
European Myeloma Network (EMN), 629
European Organization for Research and Treatment of Cancer (EORTC), 912
European Society for Blood and Marrow Transplantation, 1084
Everolimus, 630
Evidence-based medicine, 1291
Extended spectrum beta lactamase (ESBL), 1064
Extended-field radiotherapy (EFRT), 942
Extracellular domain (ED), 1249
Extracorporeal photopheresis (ECP), 1131, 1132, 1216
Extramedullary disease, 81, 365–367
Extramedullary plasmacytoma (EMP)
  clinical features, 587
  diagnostic criteria, 587
  treatment and outcome, 587–588
Extranodal marginal zone B-cell lymphoma of mucosa-associated lymphoid tissue (MALT lymphoma), 783–785
Extranodal NK/T-cell lymphoma nasal type (ENK/TL-NT), 802, 876
Eye, Waldenstrom's macroglobulinemia, 625

F
Famciclovir, 1105
Familial aggregation
  Hodgkin lymphoma, 760
  lymphoma, 755
  non-Hodgkin lymphoma, 766
Familial amyloid cardiomyopathy, 657
Familial Hodgkin lymphoma, 925
Familial predisposition
  case-control study, 732

cohort study, 732
etiology, 731–734
host genetics, 731
prognosis, 734
twin and registry-based studies, 731
Family genetic linkage study, germline genetic, 734
Fanconi syndrome, 660
Farnesyl transferase inhibitors, 392
FAS-associated death domain (FADD), 73
Fatigue, 652
Fc-fusion protein romiplostim, 1253
Febrile neutropenia, 1063, 1065, 1066, 1068, 1069, 1248
Febrile nonhemolytic transfusion, 1146
Febrile reactions, 1170
Fems-like receptor tyrosine kinase (FLT3), 382
Fetal liver cells, 1185
Fever, 686
FGFR3 gene, 647
Fibrinolysis, 442
Fibrosis MDS, 227, 492
Fine-needle aspiration, NHL, 953
FIP1L1 fusion gene, 420
FIP1L1-PDGFRA, 420
FL. *See* Follicular lymphoma (FL)
Flow cytometry, 80, 86, 240, 261, 488, 705, 829
immunophenotype, HCL, 138
minimal residual disease determination by, 256
minimal residual disease testing in CLL by, 91 *see also* Multiparameter flow cytometry (MFC)
FLU OIA assay method, 1100
Fluconazole, 1054
Fludarabine, 5, 121, 1181
Fludarabine and cyclophosphamide and rituximab (FCR), 122, 962
Fluorescence in situ hybridisation (FISH), 49, 55, 99, 119, 281, 282, 312, 427, 778, 782, 864, 868–870, 872, 875, 877–880, 882–884, 1256
CLL, 854–857
cutaneous T-cell lymphoma, 879
DLBCL, 865, 866
FL, 861, 862
lymphoplasmacytic lymphoma, 858
MALT, 859, 860
MCL, 862, 863
PMBL, 870
Fluorescence-activated cell sorter (FACS), 705
Fluorodeoxyglucose (FDG), 589, 689
Fluoroquinolones, 1069
Fluoxetine, 1297
FMS-like tyrosine kinase 3 (FLT3), 167–168
FLT3-ITD^{POS} T-lineage ALL, 248–249
inhibitor, 367, 392
mutations, 289, 392
mutation in AML, 247–248
FLT3 internal tandem duplication (*FLT3*-ITD), 166, 282
Follicular lymphoma (FL), 773, 780–782, 827, 832
centroblasts in, 781
clinical features, 952
cytogenetic analysis, 860
diffuse pattern, 781
discordance, 781
extranodal sites, 782
genetic aberrations, 861
grading cases of, 781
GWAS, 738
in situ, 782
IRF4 rearrangement, 783
molecular hallmark, 782
neoplasm, 781
pan-B-cell markers, 781
pediatric-type FL, 782
treatment, 960–962
tumor genomic, 725–726
WHO classification, 781
with 1p36 deletion, 782–783
Follicular Lymphoma International Prognostic Index (FLIPI), 958
Follicular T-cell lymphoma (FTCL), 804, 805, 880
Fostamatinib, 5
Foxhead transcription factor (FOXO), 383
Frailty
complications, 546–547
definition, 540, 541, 544
dosing and schedule, 545–546
geriatric assessment, 541–543
treatment, 544–545
Fred Hutchinson Cancer Research Center, 1187
Free light chain (FLC) assay, 532
French Polycythemia Study Group (FPSG), 467
French Society for Pediatric Oncology (SFOP), 980
French Society of Bone Marrow Transplantation and Cellular Therapy (SFGM-TC), 1186
French–American–British (FAB) classification, 376, 380, 409
Frozen red blood cells, 1141
Funagl infections, hematological malignancies, 1070–1074
Candida infections, 1070
antifungal prophylaxis, 1071
clinical presentation, 1070
diagnosis, 1071
epidemiology, 1070
risk factors, 1070
treatment, 1071
opportunistic molds, 1071, 1072
diagnosis, 1073
epidemiology, 1072, 1073
features, 1072
treatment, 1073, 1074
Fundic hemorrhage, 378
Funduscopy, Waldenstrom's macroglobulinemia, 620, 626
Fungal infections, hematological malignancies, 1063
Fusarium, 1073, 1074
F. solani, 1073
Fusion partners (FPs), MLL, 169

G

GAD *See* Generalized anxiety disorder (GAD)
Gadolinium, amyloidosis, 658, 659
Galenic theory, 676
Gamma aminobutyric acid (GABA), 1300
Gamma irradiation, 1141
Gamma-D-glutamyl-1-tryptophan, 1223
Ganciclovir, 1084–1087, 1091
Gastrointestinal tract
NHL, 956
Waldenstrom's macroglobulinemia, 624
GB virus-C (GBV-C), 1108
GCSF receptor (CSF3R), 1247
Gefitinib therapy, 430
Gemtuzumab ozogamicin (GO), 367, 386, 391, 394, 1276

Gene expression, T-ALL differentiation stages with, 250
Gene expression profiles (GEP), 647, 790
Gene–environment interaction, germline genetic, 738
Generalized anxiety disorder (GAD), 1293
Generalized Anxiety Disorder-7 scale (GAD-7), 1292
Genetic anticipation, 761, 766
Genetic lesion, 238
Genome lesion, 110
  targeting 11q23, 101–102
Genome-wide association studies (GWAS), 735
  CLL, 735
  DLBCL, 738
  follicular lymphoma, 738
  Hodgkin lymphoma, 735
  lymphoma, 736, 737, 739
Genomic instability and DNA repair, 35
Genomics, 726
  clinical practice, 741
  future direction, 742–743
  germline *see* Germline genetic
  implication, 741, 742
  prognostication, 742
  risk assessment, 741
  tumor *see* Tumor genomic
German Hodgkin Study Group (GHSG), 812, 916, 942, 943
German Multicenter Trials for Adult ALL (GMALL), 337
Germinal center (GC) microenvironment, 795
Germinal center B-cell expressed transcript-1 (GCET), 781
Germinal center B-cell like (GCB) groups, 790
Germinal center-DLBCL (GC-DLBCL), 831
Germline genetic, 734, 735, 738–740
  candidate gene studies, 735, 739
    cytokine genes, 739
    DNA and chromosomal integrity, 740
    FCGR2A/FCGR3A, 740
    GWAS, 740
    pharmacogenetics and metabolis, 740
  etiology, 734, 735, 738, 739
  familial predisposition, 731
  family genetic linkage studies, 734
  gene–environment interaction, 738
  GWAS, 735, 738
    CLL, 735
    DLBCL, 738
    follicular lymphoma, 738
    Hodgkin lymphoma, 735
  prognosis, 739–742
  risk assessment, 741
Germline polymorphism, 308
Germline syndrome, 359
Gingival hypertrophy, 376
Glucocorticoids, Hodgkin lymphoma, 695
Glucose 6 phosphate dehydrogenase (G6PD), 484
Glutathione (GSH), 424
Glutathione S-transferase (GST) genes, 486
Goldie–Coldman hypothesis, 914
Graft failure and rejection, 1226
Graft-versus-host disease (GVHD), 390, 1050, 1081, 1132, 1147, 1179, 1201, 1276, 1301, 1302
Graft-versus-leukemia (GVL) effect, 349, 1202
Graft-vs.-tumor (GVT) effect, 1180
Gram negative bacteria, 1067
Gram positive bacteria, 1064
Gram-negative bacteremia, 1053
Granisetron, 386
Granular blast, 224
Granulocyte collection and storage
  granulocytes, 1168
  gravity methods, 1168
  HES, 1167
  normal donor granulocyte transfusions, 1169
  platelet collection, 1167
Granulocyte colony-stimulating factor (G-CSF), 468, 567, 1009, 1058, 1167, 1183
  ALL, 1248
  AML, 1248
  GM-CSF, 1249–1250
  PMN, 1247
Granulocyte transfusion therapy, 1153, 1167, 1170, 1171
Granulocyte transfusions, 1059, 1169, 1170, 1219
Granulocyte–macrophage colony-stimulating factor (GM-CSF), 32, 469, 1009, 1184, 1249
  erythropoietin, 1250–1251
  MDS, 1252–1253
  prophylaxis, 1255
  thrombopoietin receptor agonists, 1251
Granulocyte–monocyte progenitor (GMP), 32, 33
Granulocytes, 1168
  transfusions, 1042, 1043
Granulocytic sarcoma, 52, 377, 378
Granulocytopenia, 344
Gravity leukapheresis, 1168
Growth factor priming, 1253–1254
GS-4059 (ONO-4059), 127
Guanosine triphosphatases (GTPases), 32
Guillain-Barré syndrome, 623
GWAS. *See* Genome-wide association studies (GWAS)
Gynecomastia, 611
HHairy cell leukemia (HCL), 92, 135–145
  clinical features
    general, 139
    infectious complications, 139
    secondary malignancies, 139–140
  cytogenetic analysis, 857
  cytology, 136–137
  diagnosis, 136
  differential diagnosis, 140
  epidemiology and etiology, 135
  histopathology
    blood and bone marrow, 137
    genetic features, 138
    spleen and liver, 137–138
  history, 135
  immunoconjugates and targeted therapy, 145–147
  immunophenotyping
    flow cytometry, 138
    immunostains, 138–139
  pathogenesis
    adhesion/homing, 136
    cytogenetics, 136
    ontogey, 136
  therapeutic pathway, 146
  treatment
    cladribine, 141–143
    evaluation and follow-up, 144–145
    general, 141

interferon, 144
pentostatin (2′-deoxycoformycin), 143
purine nucleoside analogue, 141, 145
relapsed and resistant disease, 145
splenectomy, 143–144
variant, 140–141
Handwashing, 1050
Hans classifier, 790
Haploidentical marrow transplant, 1217
Haploidentical SCT, 348
Haploidentical transplantation, 1182, 1187
HCL. *See* Hairy cell leukemia (HCL)
HCT. *See* Hematopoietic cell transplantation (HCT)
Health-care workers (HCWs), 1052, 1098
Heart, and amyloidosis, 657
Helicobacter pylori (*H.pylori*), 715–717, 784, 1011, 1014
Hematogone, 238, 265
Hematologic toxicity, 940
Hematological malignancies, 1153, 1159, 1162, 1255
immunodeficiency, 1049–1050
instructions to patients, 1051
risk factors, 1048
Hematological neoplasms, 359, 1291–1298, 1300, 1301
antidepressants, 1296
side effects, 1297
titration protocol, 1296
anxiety
adjustment disorder, 1293
anticipatory anxiety and nausea, 1294
defined, 1291, 1292
due to general medical condition, 1294
GAD, 1293
panic disorder with/without agoraphobia, 1293
prevalence, 1292
psychopharmacological treatments, 1296
risk factors, 1292
screening and measuring, 1292
social anxiety disorder, 1293
specific phobia, 1293
substance-induced, 1294
behavioral activation, 1299
benzodiazepines, 1297, 1298
burnout and compassion fatigue, 1303
cancer support groups, 1300
cancer treatments, neurocognitive effects of, 1303
cognitive therapy, 1298, 1299
collaborative care, 1304
communication skill training, 1303
delirium/confusion, 1300
clinical features, 1300
course, 1300
management, 1301
mortality, 1301
risk factors, 1300
depression
anxiety and suicide, 1296
defined, 1294
differential diagnosis, 1295
measuring and screening, 1295
medical workup, 1296
prevalence and impact, 1294, 1295
psychopharmacological treatments, 1296
risk factors, 1295
HSCT, 1301–1303
pathophysiology, 1300
problem-solving therapy, 1299
psychoeducation, 1298
psychostimulants, 1298
psychotherapy, 1298
Hematopoiesis, 167
Hematopoietic cell transplantation (HCT), 364, 573, 1052, 1193, 1211–1215, 1232
allogeneic HCT, 578
blood marrow
recipient suitability, 1212–1215
UCB, 1211
conditioning regimen, 575
criteria, 574
future directions, 580
high-dose therapy, 575–576
lenalidomide maintenance, 579
for myeloma, 573
optimal induction regimen, 576
post-HCT consolidation therapy, 579
randomized trials, 575
risk factors, 575
salvage HCT, 578–579
second malignancies, 1233–1234
stem cell procurement, 574–575
tandem transplants, 577–578
timing of, 576–577
toxicities, 576
transplant eligibility, 573–574
Hematopoietic cell transplantation-specific comorbidity index (HCT-CI), 574, 1214
Hematopoietic colony-stimulating factor (CSF), 910
Hematopoietic growth factor (HPGF), 344, 1247
Hematopoietic malignancy, WHO classification, 179
Hematopoietic stem cell (HSC), 30, 33, 81, 168, 778, 1179, 1181, 1183, 1184
characterization, 1179–1180
donors, 1181
rationale for transplantation, 1180–1181
RIC regimens, 1181
types
bone marrow *vs.* peripheral blood, 1183
UCB, 1183
Hematopoietic stem cell transplantation (HSCT), 312, 440, 697, 1058, 1093, 1153, 1158, 1179, 1302
allogeneic, 319, 322
bone marrow, 1183–1184
high-intensity (myeloablative) regimens, 1181
for t-AML, 476
treatment, 993
Hemodialysis (HD) technique, 567
Hemophagocytosis, 216
Hemorrhage, 344, 1268
Hemorrhagic cystitis, prevention and treatment, 1225–1226
Heparin, 376
Hepatic veno-occlusive disease, 1276
prothrombotic cytokines, 1276
stem cell transplantation, 1276
Hepatitis A virus (HAV), 1103
Hepatitis B core antibody (anti-HBc), 1104
Hepatitis B virus (HBV), 1056, 1103–1105
Hepatitis C virus (HCV), 617, 769, 785, 786, 1056, 1105–1108

Hepatitis G virus (HGV), 1108
Hepatitis surface antigen (HbsAg), 1104
Hepatitis testing, 1043
Hepatitis viruses, 1086, 1103
  hepatitis A, 1103
  hepatitis B, 1103–1105
  hepatitis C, 1105–1107
  hepatitis G, 1108
Hepatocellular carcinoma, 1105
Hepatomegaly, 653
Hepatosplenic candidiasis, 1070
Hepatosplenic T-cell lymphoma (HSTCL), 803, 804, 877
Hepatosplenomegaly, 210, 308, 340
Herpes simplex virus (HSV), 1055, 1079
  diagnosis, 1080
  infection patterns, 1079, 1080
  infection with acyclovir, 1081
  prophylaxis, 1080
  treatment, 1080
Herpesvirus pneumonia, 1080
Herpesviruses, 1006, 1079
HHV. *See* Human herpesviruses (HHV)
High-cutoff hemodialysis (HCO-HD), 603
High-dose chemotherapy (HDCT), 982–983
High-dose intravenous acyclovir, 1086
High-dose melphalan, 576
High-dose methotrexate (HDMTX), 986
High-dose therapy, 631
High-grade B-cell lymphomas, 798
  MYC, *BCL2*, and *BCL6* translocations, 798
  NOS, 798–799
Highly active antiretroviral therapy (HAART), 765, 1008, 1009, 1109
High-resolution chest computed tomography (CT), 1072
High-titer hemagglutinin, 1166
Histiocyte, 773, 774
Histocompatibility issues, in granulocyte transfusions, 1170
Histocompatible donor, 1155
Histologic classification, NHL, 955
Histone acetyltransferase (HAT), 502
  activity, 170, 413
  domain, 170
Histone code, 413
Histone deacylases (HDACs), 502
  enzyme activity, 413
  inhibitors, 476
Histone marks, 413
Histone methyltransferase (HMT), 107, 412
HIV-associated immunodeficiency, 1091
HIV-associated lymphomas, 1004
  clinical features, 1006
  genetic lesions, accumulation of, 1004, 1005
HL. *See* Hodgkin lymphoma (HL)
Hodgkin and Reed-Sternberg (HRS) cells, 90, 729, 836
Hodgkin lymphoma (HL), 70, 85, 109, 693, 695, 696, 755, 760, 761, 774, 809, 810, 831, 836–838, 908–915, 918–921, 942, 1089
  alcohol-induced pain, 908
  biomarker, 907
  cardiac complications, 921
  case clustering, 761–762
  chemotherapy, 912, 913
    alkylating agents, 693
    combined drug therapy, 696
    glucocorticoids, 695
    vinca alkaloids, 696
  classical, 811
  complications and associations, 925
  consolidation radiotherapy, 917–918
  conventional approach, 914
  cytogenetic analysis, 882, 884
  diagnosis, 907
  diagnostic criteria, 682–687
  early pathogenesis, 903
  early-stage, 942–944
  EBV-related lymphomas, 1002
  EBV role, 760
  environmental/occupational cause, 762
  etiology, 903–905
  extranodal, 905, 906
  familial HL, 925
  female, 758
  follow-up study, 908
  future considerations, 926
  genetic analysis, 729
  genetic susceptibility, 904
  genetics, 760–761
  GWAS, 735
  high-dose consolidation therapy, 916–917
  histopathological heterogeneity, 687–688
  historical aspects, 685–686
  HIV, 905, 1007, 1008
  immunophenotypic findings of, 813–814
  incidence, 756, 757, 759
  initial presentation, 905–906
  international incidence, 757–759
  international prognostic score, 907, 908
  long-term complications, 903
  lymphocyte predominant, 906
  lymphocyte-depleted HL, 813
  male, 758
  malignant cell, 904
  mixed cellularity, 813
  molecular genetic findings, 814
  newer intensive combination, 915–916
  nodular lymphocyte predominant, 810, 811, 884
  nodular sclerosis, 812
  orthovoltage radiation therapy, 690
  pregnancy and infertility, 924
  pulmonary complications, 922
  radiotherapy, 911, 912
  recommended pretreatment evaluation, 907
  relapse, 918
    chemotherapy, 919–920
    high-dose therapy, 920
    radiation therapy, 918–919
  renal disease, 925
  response evaluation, 908
  Richter transformation, 905
  risk factor, 762
  salvage therapy, 918
  second neoplasms, 922
  socioeconomic status, 759–760
  stage IIB bulky disease, 944
  stages, 688–689
  staging system, 907–909
  statistics, 757
  stem cell transplantation, 920–926
  surveillance, 944
  thyroid dysfunction, 925

treatment, 692, 903
early stages, 911–915
evolution, 909, 910
general strategy, 910–911
mortality/morbidity, 921
and two-disease hypothesis, 814
WHO classification, 776, 809
Hodgkin lymphoma-Richter transformation, 90
Hodgkin's disease, 1090, 1272
Homeobox (*HOX*) gene, 169
Homoharringtonine, 444
Hormone response elements (HREs), 411
Hospital water system, 1052
Host genetic. *See* Germline genetic
Host-versus-graft (HVG), 1201
HPGF. *See* Hematopoietic growth factor (HPGF)
HSC. *See* Hematopoietic stem cell (HSC)
HSCT. *See* Hematopoietic stem cell transplant (HSCT)
HSV. *See* Herpes simplex virus (HSV)
HTLV-1. *See* Human T-lymphocytotropic virus-1 (HTLV-1)
HTLV-I basic leucine zipper factor (HBZ), 801
Human germinal center-associated lymphoma (HGAL), 781
Human herpesvirus (HHV), 1092
HHV-4, 711
HHV-8, 769
Human immunodeficiency virus (HIV) infection, 999
classical Hodgkin lymphoma, 905, 906
non-Hodgkin lymphoma, 765
Human leukocyte antigen (HLA), 1193
allele-specific risks in transplantation, 1203
class I genes, 1193
class II genes, 1194
expression, 1202–1203
frequencies, 1167
haploidentical donors, 1182, 1185
HLA-haploidentical and cord blood transplants, 1216–1217
HLA-match unrelated donor (MUD), 1215
HLA-mismatched transplantation, reduced intensity conditioning, 1200, 1201
mismatching, 1199
nomenclature, 1197
type donors, 1040
typing methodology, 1194
HLA-DPB1, 1199
Human parvovirus B19, 1108
Human platelet alloantigens (HPA), 1204
Human T-cell lymphotropic viruses (HTLV), 1006, 1109
Human T-lymphotropic virus-I (HTLV-I), 713–715, 769
Hydrocele, 925
Hydroxyethyl starch (HES), 1130, 1167
Hydroxyurea, 53, 1269
Hypercalcemia, 309, 379
Hyperdiploidy, 203, 293, 311, 640, 646
Hypergammaglobulinemia, 1018, 1020
Hypergranular blast cell, 207
Hyperhistaminemia, 431
Hyperleukocytic leukostasis, 1143
Hyperleukocytosis, 284, 309, 361, 375, 1130, 1131, 1133, 1159
blast cells, 1130
definition, 1130
Hypermethylation, 108
Hyperproteinemia, 518
Hyperreactivity, in patients, 1267
Hyperuricemia, 343
Hyperviscosity syndrome, 621, 1134, 1143
Hypnosis, 1299
Hypoactive delirium, 1295
Hypocalcemia, 379
Hypocellular acute myeloid leukemia (AML), 199, 228
Hypocellular MDS, 228, 492
Hypocellular myeloid neoplasm, 228
Hypocholesterolemia, 380
Hypodiploidy, 203, 293, 312
Hypofibrinogenemia, 430
Hypogammaglobulinemia, 81, 129, 1053
Hypogranular promyelocyte, 207
Hypogranulation, 222
Hypomethylation, 108
Hyponatremia, 1297
Hypovolemia, symptoms, 1154
Hypoxia-inducible factor 1α (HIF-1α), 1250

I
iAMP21. *See* Intrachromosomal amplification of chromosome 21 (iAMP21)
Ibritumomab tiuxetan, 962
Ibrutinib, 125, 147, 148
ICSBP. *See* Interferon consensus sequence-binding protein (ICSBP)
ID3 mutation, 798
Idarubicin, 433, 437
Idelalisib, 127
Idiopathic dysplasia of uncertain significance (IDUS), 493
Idiopathic thrombocytopenic purpura (ITP), 309
IFIs. *See* Invasive fungal infections (IFIs)
Ifosfamide, cisplatin, etoposide (ICE), 1255
IGHV mutation status, 87
IgM, 621–624
Waldenstrom's macroglobulinemia
autoantibody activity, 622
cold agglutinin activity, 623
cryoglobulinemia, 622
hyperviscosity syndrome, 621
peripheral neuropathy, 622
tissue deposition, 624
monoclonal gammopathy of undetermined significance, 525, 526, 727
IKZF1 gene, 34
deletion, 312
Imatinib, 39, 40, 352, 1274
for chronic myeloid leukemia, 54–58
for pregnancy, 62
Immune dysfunction, 70
Immune dysregulation, 999
Immune reconstitution
active immunization, 1058
CSF, 1058
granulocyte transfusions, 1059
IVIG, 1058
Immune surveillance, 728
Immune thrombocytopenic purpura (ITP), 1040, 1264, 1272
Immune-mediated cytopenia, 129
Immune-mediated thrombocytopenia (ITP), 500
Immunoblast, 790
Immunodeficiency, non-Hodgkin lymphoma, 764
Immunodeficiency-associated BL, 796
Immunoglobulin (Ig), 651, 773
Immunoglobulin heavy-chain (IGH), 141

Immunoglobulin heavy-chain variable (IGHV) region, 4, 81
Immunoglobulin light chains amyloid, 652. *See also* Amyloidosis
Immunoglobulin V (IgV), 837
Immunologic abnormality, Waldenstrom's macroglobulinemia, 625
Immunological markers, 702–704
Immunomodulatory agents, 520
Immunomodulatory drugs, amyloidosis, 664
Immunoperoxidase staining, 137, 139
Immunophenotypic findings of classical HL, 813
Immunoproliferative small intestinal disease (IPSID), 783. *See also* Alpha heavy chain disease
Immunostains, 138
Immunosuppressive therapy, 501
Immunotherapy, 1040
Implantable defibrillator, 660
Increased platelet activity, 1267
Indirect antiglobulin test (IAT), 1143
Indolent NHL
  conventional salvage therapy, 965
  DLBCL, 963
  follicular lymphoma, 960
  PCNSL, 966
  small lymphocytic lymphoma, 962
Induction chemotherapy, 381, 383
Induction therapy, 433
  ATRA, 433
  history of, 433
  MM, 552
  response to, 386–388
  toxicity of, 386
Infection prevention and posttransplant vaccination, 1234
Infection prevention, hematological malignancies, 1047, 1050, 1052
  antimicrobial prophylaxis, 1053
  control measures, 1050
    handling pets, 1050
    personal hygiene, 1050
    skin and mucosal care, 1050
  environmental precautions
    hospital precautions, 1052
    household exposure, 1052
  invasive precedures, 1053
Infectious Disease Society of America (IDSA), 1067
Infectious mononucleosis, 1090
Infertility, Hodgkin lymphoma, 924
Infiltrative cardiomyopathy, 652, 654
Inflammatory cells, 686
Influenza A and B viruses, 1099
  detection, 1099, 1100
  prevention, 1101
  treatment, 1100, 1101
Influenza infections, 1099
Information technology, unrelated donor identification, 1197–1198
Inherited genetic susceptibility model, 71
Inherited immunodeficiency syndrome, 764
Inhibitor
  of B-cell receptor, 124
  PI3K, 127
  second-generation BTK, 126
Inotuzumab ozogamicin (INO), 254
Intensity modulated radiotherapy (IMRT), 940
Intensive chemotherapy, 504, 1212
Intensive postremission chemotherapy, 1157
Interferon, 144, 1091
Interferon-alpha, 54, 140, 144
Interferon consensus sequence-binding protein (ICSBP), 32
Interferon-stimulated responsive element (ISRE), 37
Interleukin-1 receptor (IL-1R) signaling, 618
InterLymph analysis, 732
Intermediate cluster region (ICR), 782
Internal tandem duplication (ITD), 207, 383
International Bone Marrow Transplant Registry (IBMTR), 1016
International Database on Hodgkin's Disease Overview Study Group, 917
International Histocompatibility Working Group (IHWG), 1201
International Lymphoma Radiation Oncology Group (ILROG), 942
International Lymphoma Study Group, 704, 775
International Myeloma Working Group (IMWG), 531, 551, 585
International Prognostic Factor Project, 907
International Prognostic Index (IPI), 739, 789, 904, 941, 958, 959, 963
International Prognostic Scoring System (IPSS), 497, 1252
International Working Group (IWG), 498
International Workshops on Waldenström's Macroglobulinemia, 626
Interstitial pneumonitis, 1084
Intestinal viruses, 1092, 1093
Intracellular domain (ID), 1249
Intrachromosomal amplification of chromosome 21 (iAMP21), 314
Intracytoplasmatic signaling, 1183
Intravascular large B-cell lymphoma (IVLBCL), 794
Intravenous haloperidol, 1301
Intravenous immunoglobulin (IVIg), 1220, 1221, 1272
Invasive aspergillosis (IA), 1054, 1072
Invasive fungal disease (IFD), 1071, 1072
  diagnosis, 1073
  features, 1072
Invasive fungal infections (IFIs), 1070
Involved field radiation therapy (IFRT), 942, 989
Involved node radiotherapy (INRT), 942
Involved site radiotherapy (ISRT), 942
Ionizing radiation, 468
IPI-145 (Duvelisib), 128–129
Ipilimumab, 840
IPSID *See* Immunoproliferative small intestinal disease (IPSID)
IRF4 rearrangement, 783
Iron chelation, 499
ISGylation, 422
IVIG. *See* Intravenous immunoglobulins (IVIG)
IVLBCL. *See* Intravascular large B-cell lymphoma (IVLBCL)
Ixazomib-lenalidomide-dexamethasone (IRD), 558

J

JAK2 mutation, 1268
Japan Marrow Donor Program (JMDP), 1199, 1201
JC human polyomavirus (JCV), 904
Joints, Waldenstrom's macroglobulinemia, 624
JUNB gene, 32
Juvenile monomyelocytic leukemia (JMML), 26, 27, 229, 420

K

Kahun gynecological papyri, 675
Kaposi's sarcoma (KS), 764, 769, 1079, 1092, 1109
Kaposi's sarcoma-associated herpesvirus-like virus (KSHV), 1008

Kataegis, 103
Kidney transplantation, 604
Kiel classification
  lymphoma, 704
  NHL, 955
Ki-67 expression, 795
Killer cell immunoglobulin-like receptor (KIR), 773
KIT mutations, 168
Klebsiella pneumoniae carbapenemase (KPC), 1064
KMT2A (MLL), 361, 365, 367
KRD. *See* Carfilzomib-lenalidomide-dexamethasone (KRD)
Krüppel-like factor 2 (*KLF2*), 420, 727, 787
Kundrat's disease. *See* Lymphosarcoma

L
Lamivudine, 1105
Lardaceous change, 651
Large B-cell lymphoma (LBCL), 783, 992
Large granular lymphocytic leukaemia, 874
Large-cell lymphoma (LCL), 130, 988
L-Asparaginase, 344, 1273
LBL. *See* Lymphoblastic lymphoma (LBL)
Lenalidomide, 501, 564, 664
  agents, 520
  PCL, 641
Lenalidomide-dexamethasone (RD), 558–559
Lenalidomide-free interval, 1184
Leptomeningeal, 978
Leri–Weill dyschondrosteosis (LWD), 761
Lesion, genome, 110
Lestaurtinib, 392
Letermovir, 1086
Leukapheresis, 53, 432, 1129, 1130. *See also* Hyperleukocytosis
Leukemia, 3, 161, 1039, 1041–1043. *See also* Acute leukemia
  early T-cell precursor (ETP), 249–250
  fusion gene, antigen for, 241
  hepatitis testing, 1043
  infections, treatment and prevention
    antiemetics, 1043
    bacterial, 1041, 1042
    fungal, 1042
    granulocytes transfusions, 1042, 1043
  lymphoblastic, 157
  and myelodysplastic syndrome, 1263, 1264
    coagulation defects, 1263–1264
    hairy cell leukemia, 1265
    platelet defects, 1264–1265
  platelet medicine, 1039–1041, *see also specific types of leukemia*
  transfusion medicine, 1039
Leukemia cutis, 377
Leukemia initiating activity (LIA), 417
Leukemia-associated immunophenotype (LAIP), 238, 264
  MFC, 261
  multitude of, 266
  stability, 267
Leukemia-free survival (LFS), 1186
Leukemia-initiating cell (LIC), 411
Leukemic blast cells, 239, 377
Leukemic cardiac infiltration, 379
Leukemic clone, 238
Leukemic promyelocytes, 442
Leukemic reticuloendotheliosis, 135
Leukemogenesis, 54, 81, 161, 170, 171, 290
  PML-RARα in, 415
Leukemogenic agents intensity, 1256
Leukemoid reaction, 49
Leukoagglutinins, 1165
Leukocytes, 1082, 1166
  adherent filters, 1155
  inactivation, UVB, 1165
  reduced red blood cells, 1140
Leukodepletion, 1165
  blood products, 1162
  leukocyte-adherent filters, 1155
  platelet products, 1155
  prestorage, 1155
  transfusion-transmitted viral disease, 1155
Leukoreduced red cells, 1148
Leukostasis, 360
Life-threatening hemorrhage, 1157
Li-Fraumeni syndrome, 293
Ligand binding domain (LBD), 411
Light-chain MGUS (LCMGUS), 526
Linkage analysis, chronic lymphocytic leukemia, 71
Linkage disequilibrium (LD), 1197
Liposomal ATRA, 435
Liver function tests (LFTs), 1104
Localized plasmacytomas, 585
Local–regional therapy, lymphoma, 940
Lonafarnib, 392
Lorazepam, 1297
Loss of heterozygosity (LOH), 99
Low transplant-related mortality (TRM), 1185
Low-dose chemotherapy, 502
LPD. *See* Lymphoproliferative disorder (LPD)
LPL. *See* Lymphoplasmacytic lymphoma (LPL)
Lucatumumab, 840
Luminex® bead-based technology, 1195
Lung, Waldenstrom's macroglobulinemia, 624
LWD. *See* Leri–Weill dyschondrosteosis (LWD)
Lymph node, 85, 678–680, 698
  chains, 905
  Waldenstrom's macroglobulinemia, 626
Lymphadenopathy, 340, 682, 905
Lymphatic system, 680
Lymphoblast, 309
  cytogenetic analysis, 244
  leukemia, 157
Lymphoblastic lymphoma (LBL), 203, 986
  clinical features, 953
  clinical presentation, 985
  Cooperative Group protocols, 987–988
  cytochemistry, 200
  genetics, 202, 203
  immunohistochemistry, 202
  molecular biology, 985
  morphologic features, 200
  pathology, 984–985
  treatment, 985–987, 992, 993
Lymphoblastoma, 698
Lymphochip, 706
Lymphocyte, 679
  classification, 704
  redistribution, 5
  inactivation, 1164
Lymphocyte predominant (LP) cells, 810
Lymphocyte-depleted HL (LDHL), 813

Lymphocyte-depleted tumors, 1003
Lymphocyte-rich classical HL (LRCHL), 811
Lymphocytosis
  monoclonal B-cell, 74, 79
  redistribution, 124
Lymphocytotoxic antibody levels, 1163
Lymphoid, 238
Lymphoid follicle, 773
Lymphoma, 675, 723, 724, 755, 858
  AIDS-associated, 1005, 1006, 1008, 1009
  autoimmune diseases, 1018–1021
  BMT
    clinical features, 1017
    epidemiology/demographics, 1016, 1017
    histopathology, 1017
    pathogenesis, 1017
  body cavity-based, 1008
  breast cancer, 1022
  CD5–/CD10–lymphoma, 830, 831
    AILD, 834
    ALCL, 834, 835
    alemtuzumab, 839
    ATLL, 835
    BL, 833
    CD5–/CD10+, 831
    CTCLs, 835, 836
    dacetuzumab, 839
    DLBCL, 831
    epratuzumab, 839
    FL, 832
    HL, 836–838
    immune checkpoint blockade, 840
    immunotherapy, 838
    ipilimumab, 840
    lucatumumab, 839
    mogalizumab, 839
    NK/T, 836
    obinutuzumab, 838
    ofatumumab, 838
    PD-1 inhibitors, 840
    PTCL, 833
    rituximab, 838
    T-cell non-Hodgkin's lymphomas, 833
  CID, 1021
    clinical features, 1021, 1026
    EBV, 1026, 1027
    histopathology, 1021, 1026
    pathogenesis, 1026
    treatment, 1027, 1028
  classification, 701, 702
  combined-modality therapy
    advantages, 941
    local–regional therapy, 940
    operator dependence, 940
    patterns of failure, 941
  Cotswold staging, 955
  cutaneous, 707–708
  cytogenetic analysis *see* Cytogenetic analysis
  dose–response, 939–940
  early-stage, 942
  genetics, 853
  germline genetic *see* Germline genetic
  GWAS, 736, 737, 739
  Helicobacter pylori-related, 715–717
  high-grade, 946
  histopathological manifestations of, 700–702
  historical concepts of, 697–700
  HIV-associated, 1004
    Burkitt's lymphoma, 1007
    clinical features, 1006
    DLBCL, 1007
    genetic lesions, accumulation of, 1004, 1005
    Hodgkin's lymphoma, 1007, 1008
    host response, 1006
  immunology, 827
    B-cell type non-Hodgkin's lymphoma, 829, 830
    BV, 840
    CAR T cells, 841
    phenotypic analysis, 827–829
    radioimmunotherapy, 841
  infectious agents
    EBV, 999, 1000
    genetic lesions, accumulation of, 1001
    host response, 1001
  intermediate-grade, 945
  involved site radiotherapy, 942
  Kiel classification, 704
  low-grade, 945
  MALT, 727, 1010
    Borrelia burgdorferi, 1012
    Chlamydophila psittaci, 1011
    H. pylori, 1010, 1011
  microscopy/examination of tissues, 677–678
  molecular abnormalities, 958
  PBMT treatment, 1018
  posttransplant lymphomagenesis, 1014, 1015
    EBV, 1015, 1016
    treatment, 1016
  primary CNS, 1007
  prognostic classification systems, 959
  radiation treatment, 690–693
  risk, 733
  risk- and response-adapted therapy, 941, 942 (*see also specific types*)
  solid organ transplantation
    clinical features, 1013, 1014
    epidemiology/demographics, 1012
    histopathology, 1013, 1014
    immunosuppressive therapy, 1012, 1013
    type of transplant, 1012
  staging evaluation, 954
  staging system, 939
  surveillance, 944
  therapy of, 708–709
  thyroiditis-associated, 1020
  tumor genomic *see* Tumor genomic
  virus- and bacteria-related, 709–713
  WHO classification, 956
  Working Formulation, 704
Lymphomagenesis, 999, 1010
Lymphomatoid granulomatosis (LyG), 792, 1003
Lymphoplasmacytic lymphoma (LPL), 92, 93, 617, 727, 728, 779, 780, 857
Lymphoproliferative disorder (LPD), 70, 999, 1273
  B-cell, 79
  chronic B-cell, 141
Lymphosarcoma, 698

LYN kinase, 31
Lysine acetylation, 413
Lysozyme, 377

M
Macrocytosis, 226, 472
Macroglossia, 653
Magnetic resonance imaging (MRI)
  amyloidosis, 658
  NHL, 979
  Waldenstrom's macroglobulinemia, 626
Maintenance therapy, 438
  Waldenstrom's macroglobulinemia, 630
Maintenance therapy, ALL, 317
Major breakpoint cluster region (M-bcr), 29
Major histocompatibility complex (MHC), 1000, 1193
Malignancy or monoclonal gammopathy of unknown significance (MGUS), 1271
Managing platelet transfusion, algorithm, 1164
Manhattan Project, 700
Manifest Destiny, 680
Mantle cell lymphoma (MCL), 80, 726, 787–789, 830, 863, 964, 965
  abnormalities, 864
  cytogenetic analysis, 862
Mantle field, 943
Marginal zone lymphoma (MZL), 830
  B-cell, 726, 727, 738
  cytogenetic analysis, 860
Maribavir, 1085, 1086
Marrow miucroenvironment, 620
Matrix metalloproteinase-9 (MMP9), 904
Mature B-ALL, 351
Mature B-cell neoplasms, 853, 856–858, 860, 862, 865, 868–873
  ALK-positive large B-cell lymphoma, 870
  B-PLL, 856
  Burkitt lymphoma, 871, 872
  chronic lymphocytic leukaemia, 853
  CNS DLBCL, 868
  DLBCL, 865, 872, 873
  EBV-positive DLBCL, 869
  follicular lymphoma, 860
  hairy cell leukaemia, 857
  Hodgkin lymphoma, 873
  LPL, 857
  MALT, 858
  mantle cell lymphoma, 862
  nodal MZL, 860
  PMBL, 869
  primary cutaneous DLBCL, 869
  SLL, 853
  SMZL, 857
  THRLBCL, 868
Mature plasmacytoid dendritic cell proliferation (MPDCP), 220
Mature T- and NK-cell neoplasms, 873–882
Mayo Clinic Group, 656, 661, 663
MBL. *See* Monoclonal B-cell lymphocytosis (MBL)
MCL. *See* Mantle cell lymphoma (MCL)
MD Anderson Cancer Center, 1157
MDS. *See* Myelodysplastic syndrome (MDS)
Mediastinal lymph nodes, 814
Mediastinal lymphadenopathy, 905
Mediastinal masses, 310
Mediastinoscopy, 909
Mediterranean lymphoma *See* Immunoproliferative small intestinal disease (IPSID)
Megakaryoblast, 210, 218
Megakaryocyte, 213, 377
  marker, 224
Megavoltage (MV) therapy, 693
MEK inhibition, 146–147
Melphalan, 664
Memorial Sloan-Kettering Cancer Center, 699
Memory B-cell (MBC), 108
6-Mercaptopurine (6-MP), 317
Methicillin-resistant *Staphylococcus aureus* (MRSA), 1064
Methotrexate, 339, 346, 347, 438
Methyl-binding proteins (MBDs), 416
Methylphenidate, 1298
MFC *See* Multiparameter flow cytometry (MFC)
MGUS *See* Monoclonal gammopathy of undetermined significance (MGUS)
Micromegakaryocytes, 472
MicroRNA (miRNA)
  abnormal regulation of, 486
  cluster, 102
Microscopy, lymphoma, 677–678
Microvascular disease, 1269
Midostaurin, 392
Milan Cancer Institute, 913, 914
Mild allergic transfusion reactions, 1146
Miller-Fisher syndrome, 623
Mini-flow bead platform binding, 1164
Minimal change nephrotic syndrome (MCNS), 925
Minimal disseminated disease (MDD), 978, 987
Minimal residual disease (MRD), 5, 138, 144, 256, 257, 259, 260, 265, 266, 343, 344, 364, 978, 987
  in CLL, 129
  complete remission, 257–258
  detection, 343
    bone marrow and blood for, 258–259
    infections, 344
    metabolic abnormalities, 343–344
    supportive care, 343
  determination
    by flow cytometry, 256–257
    sample quality conundrum, 259–260
  levels, 322
  MFC-MRD detection, 264–265
    in B-lineage ALL, 265
    vs. molecular MRD, 261–262
    in myeloid leukemia, 266
    in T-lineage ALL, 266
  positivity and negativity, 262–263
Minimal residual disease detection (MRD), 91, 580
Minor breakpoint cluster region (M-bcr), 29
Mirtazapine, 1297
Mismatched unrelated donors, 1182
Mitogen-activated protein kinase (MAPK), 423, 1001
Mixed cellularity Hodgkin lymphoma (MCHL), 813
Mixed passive hemagglutination test (MPHA), 1204
Mixed-lineage leukemia (MLL), 169, 170
  fusion, 170
  fusion partner, 169, 170
  gene, 169
Mixed-phenotype acute leukemia (MPAL), 221, 238, 311, 395

MM. *See* Multiple myeloma (MM)
MMSET gene, 647
Mobilized peripheral blood, 1255
Modafinil, 1298
Modal number (mn), 290
Modifiable risk factors, SOS, 1224
Modification of Diet in Renal Disease (MDRD), 602
Moesin (*MSN*), 808
Mogalizumab, 839
Molecular biology
  ALCL, 989–990
  Burkitt lymphoma, 981
  DLBCL, 988
  lymphoblastic lymphoma, 985
  NHL, 978
Molecular genetic findings of classical HL, 814
Monoclonal antibody, 138, 521, 705, 708, 992
  CD33, 254
  in vivo therapy with, acute leukemia, 252
Monoclonal B-cell lymphocytosis (MBL), 74, 79, 80, 117, 777
Monoclonal gammopathy of undetermined significance (MGUS)
  clinical evaluation, 529
  clinical recommendations, 528–529
  comorbidities, 528
  definition, 525
  diagnosis, 525, 526
  epidemiology, 526
  etiology, 526–527
  genetic landscape, 528
  non-IgM MGUS, 527
  PETHEMA group, 527
  risk assessment and progression, 527–528
  scoring systems, 527
  types, 526
Monoclonal light chain protein, 659
Monoclonal protein detection, 515
Monoclonality, 518
Monocyte chemoattractant protein 1 (MCP-1), 602
Monocytoid B-cell lymphoma, 785
Monomorphic epitheliotropic intestinal T-cell lymphoma (MEITL), 803
Morphologic dysplasia, 212
  vs. cytopenia, 226
Mucosa-associated lymphoid tissue (MALT), 717, 769, 951, 1010
  Borrelia burgdorferi, 1011, 1012
  Chlamydophila psittaci (CP), 1011
  cytogenetic analysis, 858
  H. pylori, 1010, 1011
  translocations, 858
Mucositis, 1222–1223
Multicentric Castleman's disease, 1010
Multidrug-resistant organisms (MDROs), 1064
Multilineage dysplasia, 212
Multimodality approach, Hodgkin lymphoma, 910
Multi-organ failure (MOF), 1223
Multiparameter flow cytometry (MFC), 257–259, 261, 263–266
  assay, 264
  detection, 265
    myeloid leukemia, 266
    T-lineage ALL, 266
  determination, 263
  vs. molecular MRD, 261
Multiple hemostatic abnormalities, 1270
Multiple lymphomatous polyposis, 787
Multiple myeloma (MM), 511–518, 539, 559–563, 645, 652, 661, 662, 1185, 1275
  AL amyloidosis, 604
  alkylating agents, 519
  anti-DKK1, 599
  autologous bone marrow transplantation, 520
  bone disease, 566–567
  bone remodeling, 596
  bortezomib, 565, 601
  case study
    Bence Jones, 513–515
    McBean, 511–513
    Newbury, 516–518
  chromosomal changes, 646–647
  combination chemotherapy, 519
  corticosteroids, 519
  cytogenetic abnormalities, 646
  denosumab, 599
  diagnosis, 539
  diagnosis and risk stratification, 551–552
  diagnostic tests, 516
  in frail patients (*see* Frailty)
  genetics, 646
  GEP, 647–648
  geriatric assessment in, 541–542
  good/standard risk features, 647
  high-dose therapy with standard-dose therapy, 560
  high-risk features, 646–647
  imaging, 597–598
  immunomodulatory agents, 520
  induction therapy, 552–556
  infections, 566
  International staging system, 645
  IRD, 558
  kidney transplantation, 604
  KRD, 557
  lenalidomide, 520, 564, 565
  lenalidomide and bortezomib maintenance, 564
  life expectancy, 539
  magnetic resonance imaging, 598
  monoclonal antibodies, 521–522
  neuropathy, 600
  next generation sequencing, 648
  in nineteenth and twentieth centuries, 518
  osteoblast suppression in, 597
  PAD, 557
  pathogenesis, 602
  pathophysiology, 595–597
  patient's conditions, 645
  peripheral neuropathy, 566
  plasma cell, 518–519
  post-transplantation maintenance and consolidation, 563–564
  posttransplant consolidation therapy, 565–566
  preclinical models, 597
  proteasome inhibitors, 521
  proteinuria and monoclonal protein detection, 515–516
  radiation therapy, 600
  radiculopathy, 600–602
  RD, 558
  reciprocal interactions, 596
  renal dysfunction in, 602
  renal injury in, 602
  renal insufficiency, 602, 603
  revised-international staging system, 647
  risk stratification systems, 552
  stem cell transplantation, 520, 559

allogeneic transplantation, 563
ASCT in, 559–561
eligibility criteria, 559
optimal conditioning regimen, 562–563
single-with double-autologous, 562
stem cell mobilization, 563
tandem transplantation, 561–562
TD, 558
thalidomide, 601
thalidomide embryopathy, 520–521
three-drug combinations, 553–554
thromboembolic phenomena, 566
thromboprophylaxis for, 600
treatment, 519, 539, 598–600
tumor burden, 645–646
tumor clone, 646
two-drug combinations, 555
urethane distraction, 519
in USA, 551
VCD, 556
VD, 558
VRD, 556
VTD, 556
Multiplex ligation-dependent probe amplification (MLPA), 99
Multipotent mesenchymal stromal cells (MSCs), 1211
Multipotential stem cell, 773
Mutated gene
cancer, 104
in CLL, 101, 105
Mutation, 38–40, 104–106
in acute leukemia, 162
acute myeloid leukemia with gene, 211
BCR-ABL1, 38–40
CEBPA gene, 168
Class I (activating) mutation, 161, 162
Class II, 161, 162
CLL, 81
internal tandem duplication, 207
noncoding, 107
NOTCH1, 104–106, 111
in protein, 167
in Ras pathway, 363
SF3B1, 106
somatic gene, 106 (*see also specific types of mutation)*
tyrosine kinase domain, 167
MYC translocations, 798
Mycobacteria, 1057
Mycobacterium tuberculosis, 689
Mycophenolate mofetil (MMF), 1221, 1228
Mycosis fungoides (MF), 707, 730, 835, 839, 878, 879
MYD88 mutation, 526, 618, 620, 780
Myeloblasts, 204, 492
Myelodysplasia, 35, 228, 360, 483
Myelodysplastic syndrome (MDS), 171, 212, 218, 221–225, 360, 384, 432, 1181, 1187, 1250, 1252, 1265
autoimmune disorders, 486
bone marrow, 224, 484, 497
in children, 228
chronic GVHD, 505
classification, 225, 487
clinical and prognostic features, 497–498
clinical course, 483
clinical development, 485
clonal cytogenetic abnormality, 489, 490
clonal origin, 484
complications from, 483
criteria for, 494
cytogenetic analysis, 484
cytogenetic scoring system, 498
cytogenetic studies, 483
cytotoxic drugs, 486
deferoxamine, 500
diagnosis, 483, 487–492
differential diagnosis, 490
DNA methyltransferase inhibitors, 503
epigenetic alterations, 485
etiology, 484
with excess of blasts (MDS-EB), 227
with excess blasts 1 and 2 (MDS-EB1 and MDS-EB2), 495
FAB classification, 487
with fibrosis, 227, 492
fibrosis and hypocellular MDS, 492–493
FISH analysis, 489
genetic predisposing factors, 486
hematologic malignancies, 483
hematopoietic microenvironment, 485
IDUS, 493–494
immunophenotyping, 491
immunoregulatory abnormalities, 486
immunosuppressive therapy, 501
intensive chemotherapy, 503–504
IPSS-R scoring system, 498
iron toxicity, 499
with isolated del, 227, 228, 495, 496
lenalidomide, 501–502
low-dose chemotherapy, 502–503
molecular genetics, 505–506
morphologic characteristics, 222–225
morphologic features, 489
with multilineage dysplasia (MDS-MLD), 226, 495
with multilineage dysplasia and ring sideroblasts (MDS-RSMLD), 227
Nagasaki atomic bomb survivors risk, 484
neutrophil function, 496
pathogenesis, 484–487
phase III randomized studies, 503
platelet function, 496–497
presumptive evidence, 490
recurrent mutated genes in, 484
red cells/anemia, 496
with ring sideroblast single-lineage dysplasia (MDS-RSSLD), 226
with ring sideroblasts (MDS-RS), 494
separation from AML, 483
with single lineage dysplasia (MDS-SLD), 226, 495
somatic mutations, 485
stem cell transplantation, 504–505
supportive therapy, 499–500
therapy-related MDS, 486
treatment, 498–499
unclassifiable (MDS-U), 493
WHO classification, 483, 487, 488
Myelofibrosis, 197
Myeloid antigen co-expression, 310–311
Myeloid antigen-positive ALL, 342
Myeloid blast, 368
Myeloid differentiation primary response protein 88 gene (MYD82), 904
Myeloid growth factors
adverse effects on leukemia, 1254
induction, 1253
intensive post-remission chemotherapy, 1253
prophylactic use, 1252

Myeloid leukemia, 203–206, 238
classification, 204–205
cytochemistry
electron microscopy, 206
esterases, 206
myeloperoxidase and Sudan Black B, 205–206
periodic acid-Schiff reaction, 206
immunohistochemistry, 206
MFC-MRD detection in, 266
Myeloid neoplasm
classification, 362
hypocellular, 228
therapy-related, 212–213
Myeloid sarcoma, 219, 220, 365
Myeloid transcription factor, 168–169
Myeloid-associated marker, 210
Myeloma and amyloid abnormalities, 1271
Myeloma cast nephropathy (MCN), 602
Myeloperoxidase (MPO), 199, 200, 205, 206, 210
Myelopoiesis, transient abnormal, 289
Myeloproliferative disorders, 1268, 1272
Myeloproliferative neoplasm (MPN), 226, 491–493, 1265
abnormalities, 1267
bleeding and thrombosis, 1265–1266
clinical laboratory tests, 1268
decreased platelet function, 1267
increased platelet activity, 1267–1268
laboratory tests of thrombophilia, 1268
platelet abnormalities, 1267
platelet dysfunction, mechanisms of, 1266
pregnancy, 1269
Myelosuppression, 1087
Myelosuppressive chemotherapy, 1250
MZL. *See* Marginal zone B-cell lymphoma (MZL)

N
NAD(P)Quinone oxidoreductase (NQO1) gene, 486
NADPH oxidase activity, 424
Naive B-cell (NBC), 108
Narcotics, 1301
National Cancer Act, 699
National Cancer Institute and Nordic Myeloma Study Group (NCI/NMSG), 527
National Cancer Institute of Canada, 912
National Comprehensive Cancer Network, 945
National Heart, Lung and Blood Institute (NHLBI), 1219
National Institutes of Health (NIH), 1227
National Marrow Donor Program (NMDP), 1198
Natural killer (NK) cells, 703, 704, 776, 827, 999, 1003, 1083, 1194
Natural killer/T-cell lymphoma (NKTCL), 738, 836
Nausea, 1217, 1218, 1294
NB4 cells, 427
Near-tetraploidy, 293
Near-triploidy, 293
Necrotizing enterocolitis, 379
N-Ethyl-*N*-nitrourea (ENU), 161
Neoplasm, second malignant, 321
Neotope antibody, 666
Nephrotic syndrome, 660, 661
Neurocognitive late effects, 320
Neuropathy, 600
Neutropenia, 499, 1053
Neutropenic fever, 1063, 1064
chemotherapy, 1252
risk factors, 1251, 1252
Neutropenic leukemia, 1081
Next-generation sequencing (NGS), 99, 258, 648, 724
NF-kB signaling pathway, 107, 1011
N-formyl-Met-Leu-Phe (fMLP), 1247
NHL. *See* Non-Hodgkin lymphoma (NHL)
Nilotinib, 40, 58
N-methylthiotetrazole side chain, 1274
Nodal marginal zone lymphoma (NMZL), 727, 785, 786
Nodular lymphocyte predominant Hodgkin lymphoma (NLPHL), 774, 810, 836, 868
cytogenetic analysis, 884
differential diagnosis from, 792
Nodular sclerosis Hodgkin lymphoma (NSHL), 812, 813, 906
Nonalloimmunized patients, 1162
Noncoding mutations, 107
Non-COX selective nonsteroidal anti-inflammatory drugs, 1268
Non-culture-based techniques, 1071
Non-Hodgkin lymphoma (NHL), 70, 340, 755, 773, 774, 828, 945, 951, 952, 960, 962–966, 975, 999, 1001, 1004, 1005, 1012, 1018, 1019, 1079, 1080, 1088, 1089, 1109, 1251
age group, 957
AIDS, 765, 766, 1006
anti-CD20 antibody therapy, 962
B-cell type, 829
BRIGHT study, 961
Burkitt lymphoma, 768
case-control study, 732
characteristics, 994
childhood *see* Childhood non-Hodgkin lymphoma (NHL)
classification, 701, 774, 775
clinical presentation, 952–953
diagnosis, 953–954
environmental cause, 769
evaluation, 954–955
familial aggregation, 766
gastrointestinal group, 956
histologic types, 952
histopathology, 955–956
HIV-associated, 1006
ibritumomab tiuxetan, 962
immunodeficiency, 764–765
incidence, 756, 757, 762–764
indolent histologic subtypes, 960
conventional salvage therapy, 965–966
DLBCL, 963–965
follicular lymphoma, 960
PCNSL, 966
small lymphocytic lymphoma, 962
infectious agents, 769
international incidence, 763
international prognostic indices, 958–959
laboratory analysis, 957–958
occupational exposure, 767–768
origin/normal counterpart, 773
ProMace-MOPP, 961
risk factor, 770
sites of disease, 956
staging procedures, 954
staging system, 979
statistics, 763
T-cell, 773, 799, 833
treatment, 959–960
tumor bulk, 957
ultraviolet exposure, 766
uncommon classifications, 966
Waldeyer's ring, 953
WHO classification, 977
Working Formulation, 774, 775

Nonidentical haplotype, 1166
Non-IgM MGUS, 525
Non-leukoreduced random donor platelet transfusion, 1162
Nonmyeloablative (NMA), 1216
Non-nucleoside reverse transcriptase inhibitors (NNRTIs), 1008
Nonspecific esterase (NSE), 216
Nonsteroidal anti-inflammatory drugs (NSAIDs), 187, 603
Nonwoven polyester filters, 1155
No-prophylaxis policy, 1160
Nordic Myeloma Study Group, 579
North American Intergroup, 914
Not otherwise specified (NOS), 865–868, 879, 880
NOTCH1 gene, 104, 111, 314
Novel therapeutics, Waldenstrom's macroglobulinemia, 630
NPM. *See* Nucleophosmin (NPM)
NPM1 (nucleophosmin) (5q31) mutation, 250, 251, 289
Nuclear bodies (NB), 414
Nuclear export signal (NES), 166
Nuclear factor-KB (NFKB), 856, 868
Nuclear localizing sequence (NLS), 415
Nucleophosmin (NPM), 166, 167, 808
Nucleoside analogue therapy, 627
NUP98 (11p15.5) gene, 288

O
Obinutuzumab, 123, 838
Occupational exposure
  Hodgkin lymphoma, 762
  non-Hodgkin lymphoma, 767
Ofatumumab, 123, 838
Oligonucleotide array technology, 1195
Omacetaxine mepesuccinate, 59–60
Ondansetron, 386
Ontogey, hairy cell leukemia, 136
Open reading frame (ORF), 415
Ophthalmoscopy, 622
Optical microscope, 677
Optimal donor, allogeneic HCT, 1214–1215
Oral alkylating agents, 626
Oral mucositis grading, 1222
Oregon Health Sciences University, 663
Orthostatic hypotension, 652, 653
Orthovoltage radiation therapy, 690
Oseltamivir, 1100, 1101
Osteolytic skeletal lesion, 210
Osteonecrosis, 321, 612
Overall survival (OS), 1212
Overlap syndrome, 1230

P
Packed red blood cells (pRBCs), 1154
PAD. *See* Bortezomib-Doxorubicin-Dexamethasone (PAD)
Paediatric-type FL (PTFL), 861
Paired box protein 5 (PAX5), 169
Palpable lymphadenopathy, 953
Pancytopenia, 386
Panic attacks, 1293
Papilledema, 611
Papovaviruses (polyomaviruses), 1108
Paraimmunoblasts, 778
Parainfluenza viruses (PIVs), 1101, 1102
  incidence and outcomes, 1102
Paralytic poliomyelitis, 759
Paraplegia, 978
Paraproteinemia, 1270
Paroxetine, 1297
Paroxysmal nocturnal hemoglobinuria (PNH), 1141, 1270
Partial response with lymphocytosis (PRL), 124
Partial thromboplastin time (aPTT), 1271
Parvoviruses, 1108
PAS reaction, 198, 202, 216
Passenger lymphocyte syndrome, 1221
Passive immunization, 1058
Pathophysiology, 1300
Patient Health Questionnaire-9 (PHQ-9), 1295, 1296
PAX5 gene, 169
PBL. *See* Plasmablastic lymphoma (PBL)
PCL. *See* Plasma cell leukemia (PCL)
PCNSL. *See* Primary central nervous system lymphoma (PCNSL)
PCR. *See* Polymerase chain reaction (PCR)
PD-1 inhibitors, 840
Pediatric leukemia, 181
Pediatric nodal marginal zone lymphoma, 786
Pediatric Oncology Group (POG), 943–944
Pediatric-type FL, 782
Pegylated G-CSF, 1248
PEL. *See* Primary effusion lymphoma (PEL)
Pelger–Huët anomaly, 488
Pentostatin, 121, 143
Pentostatin, cyclophosphamide, and rituximab (PCR), 122
Perforin gene, 808
Periorbital purpura, 653
Peripheral blood (PB) film, 22, 23, 25, 26
Peripheral blood mononuclear cells (PBMCs), 835, 1015
Peripheral blood stem cell (PBSC), 470, 1184
Peripheral blood stem cell transplantation (PBSCT), 910, 916
Peripheral blood *vs.* marrow source, 1215–1216
Peripheral neuropathy, 622, 665, 666
Peripheral T-cell lymphoma (PTCL), 730, 731, 834, 839, 966
Peripheral T-cell lymphomas NOS (PTCL NOS), 804, 805, 879
Peripherally Inserted Central Catheters (PICCs), 1066
Peroxidase stain, 204
Personal hygiene
  food handling, 1050
  handling pets, 1050
  handwashing, 1050
  skin and mucosal care, 1050
Petechiae, 377
Pharmacologic immunosuppression, 764
Philadelphia (Ph) chromosome, 3, 9, 49, 294, 295, 342
Phlogiston theory, 681
PI3K inhibitor, 127
Plasmablastic lymphoma (PBL), 795, 1003
Plasma cell, 518
  clonal population, 654
  light chain amyloidosis, 654
Plasma cell-directed chemotherapy, 661
Plasma cell leukemia (PCL), 552, 639
  bortezomib, 641
  clinical presentation, 639–640
  diagnosis, 640, 641
  epidemiology, 639
  future directions, 643, 644
  HDT, 639
  induction regimens, 641
  lenalidomide, 641
  prognosis, 643
  role of transplant, 642–643
  treatment, 641, 642

Plasma lipid hydroperoxides, 434
Plasmapheresis, 1129
  cryoglobulinemia, 1134
  hyperviscosity syndrome, 1134
Plasminogen, 442, 1264
Platelet and granulocyte transfusion, 1154, 1160–1162
  alloimmunization
    AML, 1162
    antibodies development, 1162
    prevention, 1164–1165
  assessment of transfusion
    posttransfusion recovery, 1161
    therapeutic platelet transfusions, 1160
  cell component transfusion, 1153
  dose–response phenomenon, 1163
  granulocyte transfusion therapy, 1153
  leukodepletion, 1155
  liquid nitrogen temperatures, 1156
  platelet collection
    apheresis donor-related issues, 1154
    apheresis machines, 1154
    pooled platelets, 1154
    pRBCs, 1154
    PRP, 1154
    single donor platelets, 1154
  platelet cryopreservation, 1156–1157
  platelet storage, 1155–1156
Platelet cryopreservation
  freezing methodology, 1156
  marrow and stem cell cryopreservation, 1156
  plateletpheresis, 1156
Platelet inhibitors, 1268
Plateletpheresis/plasma exchange, 1129
Platelet prostaglandin synthesis, 1267
Platelet-rich plasma (PRP), 1153, 1154
Platelet storage
  bag qualities, 1156
  functional platelet, 1156
  lesion, 1156
  oxidative metabolism maintenance, 1156
  whole blood donations, 1155
Platelet survival, 1160
Platelet suspension immunofluorescence test (PSIFT), 1204
Platelet transfusion, 381, 1039–1041, 1153, 1165, 1166
  clinical conditions affecting, 1158
  hazards
    risk mitigation protocol, 1166
    satellite dyskeratosis, 1167
    TRALI, 1166
    transfusion reactions, 1165
  management algorithm, 1165
Pleomorphic MCL, 788
PLL. *See* Prolymphocytic leukemia (PLL)
PMBL. *See* Primary mediastinal large B-cell lymphoma (PMBL)
PML. *See* Promyelocytic leukemia (PML)
PML-RARα
  dominant repressor effect of, 418
  gain-of-function activity, 418
  homodimers, 417
  in leukemogenesis, 415–419
  PML-RARα*K160R*, 419
  proteolytic degradation, 416
  proteolytic degradation of, 421
  in response to treatment, 421–425
  SMRT from, 423
  structure and generation, 415
  substantial degradation of, 421
Pneumocystis cariini, 1232
Pneumocystis jirovecii, 1057
POEMS syndrome
  algorithm, 613
  bone marrow biopsy, 612
  Castleman disease variant of, 609
  classic findings, 611
  clinical and laboratory presentation, 610–612
  diagnosis, 609–610
  with disseminated bone marrow involvement, 613–614
  features, 609
  lenalidomide, 614
  monitoring response, 614
  pathogenesis, 609
  pulmonary hypertension, 612
  respiratory complaints, 612
  symptoms of disease, managing, 614
  treatment, 612
  without disseminated bone marrow involvement, 613
Polyclonal B-cell mitogen, 99
Polycomb group (PcG), 170
Polycythemia vera (PV), 1265
Polycythemia Vera Study Group (PVSG), 467
Polyene, 1074
Polygenic model
  of disease susceptibility, 71
  of inheritance, 71
Polymerase chain reaction (PCR), 1081, 1084, 1100, 1142, 1225
Polymerization of fibrin, 1271
Polymorphonuclear leukocyte (PML), 222
Polymorphonuclear neutrophil (PMN), 1247
Polysumoylation, 423
Ponatinib, 40, 59
Popcorn cells, 811
Positron emission tomography (PET), 923
  Hodgkin lymphoma *see* Hodgkin lymphoma (HL)
  lymphoma, 688
Post-bone marrow transplant lymphoproliferative disease (PBMT-LPD), 1016, 1018
Posterior iliac crests, 1183
Post-HCT consolidation therapy, 579
Postremission chemotherapy, 434
Posttransfusion neutrophil increment, 1219
Posttransfusion platelet counts, 1161
Posttransfusion platelet increments and refractoriness, 1158
Posttransplant consolidation therapy, 565
Posttransplant lymphomagenesis, 1014, 1015
  EBV, 1015, 1016
  treatment, 1016
Posttransplant lymphoproliferative disorder (PTLD), 976, 1001, 1003, 1013, 1014, 1056, 1091
Posttransplantation cyclophosphamide (PTCy), 1187
Preferentially expressed antigen of melanoma (PRAME), 428
Pregnancy
  APL and, 440
  Hodgkin lymphoma, 924
  imatinib for, 62
Pretarget radiolabeled antibodies, 1181
Pretransplant assessment of mortality (PAM), 1214
Primary central nervous system lymphoma (PCNSL), 728, 729, 765, 966, 967
Primary cutaneous ALCL, 990
Primary cutaneous DLBCL (PC DLBCL), 869
Primary effusion lymphoma (PEL), 795, 796, 1008

Primary mediastinal large B-cell lymphoma (PMLBL), 792, 793, 799, 831, 869, 988
Primary myelofibrosis (PMF), 1265
Primary plasma cell leukemia (pPCL), 639
Problem-solving therapy, 1299
Procarbazine, 696, 697
Prognostic gene expression, 247
Programmed death 1 (PD-1) ligand pathway, 904, 905
Programmed death ligand (PDL), 793
Proliferating cell nuclear antigen (PCNA), 1003
Prolymphocyte, 82, 85, 778
Prolymphocytic leukemia (PLL), 130, 141
Prolymphocytic transformation, 130
ProMace-MOPP, 961
PromyeLocyte (PML), 204, 410, 492
  abnormal, 207
  homodimers, 414
  hypogranular, 207
  nuclear bodies, 413–415
  nuclear localizing sequence, 415
  physiological function, 414
  protein\:protein interactions, 418
Promyelocytic leukemia (PML), 38
Prophylactic platelet transfusions, 1158, 1160
Prophylaxis, 1053
Prostate cancer, 432
Protease inhibitors (PIs), 1008
Proteasome inhibitor, 521
  Waldenstrom's macroglobulinemia, 628
Protein
  basic-leucine zipper (bZIP) domain, 168
  mutation in, 167
  targeting antiapoptotic, 124
Protein kinase C-delta (PRKCD), 426
Proteinuria detection, 515
Prothrombotic cytokines, 1276
Proton therapy, 944
Pseudodiploidy, 290
Pseudofollicle, 85, 92
Pseudolymphomas, 783, 1018
Pseudomonas aeruginosa, 1064, 1068
Pseudomonas fluorescens, 1146
Pseudomonas putida, 1146
Pseudotumor cerebri, 368, 436
Psychoeducation, 1298
Psycho-oncology programs, 1291
Psychosocial cancer care, 1291
Psychostimulants, 1298
Psychotherapy, 1298, 1300
Pulmonary complications, Hodgkin lymphoma, 922
Pulmonary distress syndrome, 434, 1072
Pulmonary hilar lymph nodes, 905
Pulmonary leukostasis, 434
Pulmonary nodule, 656
Pure red cell aplasia (PRCA), 129
Purine analogs, 121
Purine nucleoside analogue, 135, 139, 141, 144, 145

Q
Quality of life (QOL) after HCT, 1233

R
RAC guanosine triphosphatases (GTPases), 32
Radiation therapy, 909, 910, 980
Radiculopathy, 600–602
Radioimmunotherapy, 841, 1181
Radioiodine (I-131), 468
Radiolabeled CD20 antibodies, 1181
Radiotherapy, 918, 939–942, 944–946
  Hodgkin lymphoma, 911, 912, 942
  lymphoma, 690–693
    combined-modality therapy, 940, 941
    dose–response, 939–940
    early-stage, 942
    high-grade, 946
    intermediate-grade, 945
    involved site/node radiotherapy, 942
    low-grade, 945
    risk- and response-adapted therapy, 941
    stage IIB bulky disease, 944
    staging of, 939
    surveillance, 944
  NHL, 945
RARα gene. *See* Retinoic acid receptor-alpha (RARα) gene
Razoxane, 467
RD. *See* Lenalidomide-Dexamethasone (RD)
Reactive oxygen species (ROS), 423
Real-time quantitative PCR (RQ-PCR), 428, 429
Rebulla study, 1158
Receptor tyrosine kinase (RTK), 167
Recipient alloimmunization, 1170
Recipient suitability, 1213–1214
Recombinant human GM-CSF (sargramostim), 1250
Recombinant immunotoxins, 147
Red blood cell (RBC), 1129, 1130, 1139–1143, 1145–1148, 1215
  ATP, 1144
  autoantibodies, 1143
  autoimmune hemolytic anemia, 1143
  blood storage, adverse effects, 1149
  bone marrow/progenitor cell transplantation, 1143
  CMV, 1142
  components of, 1139, 1140
    frozen, 1141
    leukocyte reduced, 1140
    washed, 1140
    white blood cells, 1139, 1140
  2,3-DPG, 1144
  erythropoiesis stimulating agents, 1144
  exchange, 1133
  gamma irradiation, 1141
  preservation and storage, 1144
  storage byproducts, 1145
  transfusion, 1139, 1160
    adverse effects, 1145
    hyperviscosity, 1143
    indications, 1142
  transfusion-associated circulatory overload, 1146
    iron overload, 1146
    transfusion reactions, 1146, 1147
    transfusion-transmitted diseases, 1147
  transfusion-associated graft-versus-host disease, 1147
    alloimmunization, 1148
    immunomodulation, 1148
Redistribution lymphocytosis, 124
Reduced-intensity conditioning (RIC), 1181, 1214
    see also Nonmyeloablative (NMA)
Reed–Sternberg cell, 83, 686, 687, 689, 903, 904
  EBV and, 903
  IL13 production, 904
  MMP9, 904

Reference-strand conformational analysis (RSCA), 1194
Refractory alloimmunized patients, 1163
Refractory cytopenia of childhood (RCC), 226, 228
Reinduction therapy, ALL, 322
Relapse, 918, 919
  APL, 440
  Hodgkin lymphoma, 918
    chemotherapy, 919
    radiation therapy, 918
Relative risk (RR), 179
Relaxation training, 1299
Remission induction therapy, ALL, 318
Renal amyloidosis, 659, 660
Renal disease
  Hodgkin lymphoma, 925
  in MM, 602
  Waldenstrom's macroglobulinemia, 624
Renal dysfunction, 379, 642
Renal insufficiency, 602–604, 652
Renal replacement therapy (RRT), 603
Resistance, tyrosine kinase inhibitor, 38
Resolving infection in neutropenica with granulocytes (RING) study, 1219
Respiratory syncytial virus (RSV), 1094, 1095
  diagnosis, 1095
  prevention, 1098
  treatment, 1095–1098
Respiratory viral infections, 1056, 1094
Response-adapted therapy, lymphoma, 941
Restriction fragment length polymorphism (RFLP), 1194
Reticuloendothelial system, 686
Reticuloendotheliosarcoma, 700
Reticulosarcoma, 700
Reticulum cell, 700, 701
Retinal leukemic infiltration, 378
Retinoblastoma 1 *(RB1)* tumor, 778
Retinoic acid (RA), 167
Retinoic acid receptor (RAR), 163
Retinoic acid receptor alpha (RARα), 163, 207, 410, 411
  alternative, 419–421
  fusion gene transcript, 419
  gene, 163
  hetero-oligomeric complex, 419
  PLZF-RARα, 419–420
  signaling pathway, 163
  structure, 411
Retinoic acid response elements (RAREs), 163, 411, 413
Retinoic acid toxicity, 435–436
Retinoid X receptors (RXR), 163
Retinoids, 435
Retroviruses, 1108, 1109
Reverse transcriptase-polymerase chain reaction (RT-PCR), 49, 282, 312, 427, 428
Revised European-American Classification of Lymphoid Neoplasm (REAL), 704, 775, 955, 976
Revised International Staging System (rISS), 551
Ribavirin, 1096, 1098
Ribosomal lamellar complex, 136
Richter transformation (RT), 83, 88, 905
Richter's syndrome (RS), 6, 109, 110, 130, 955
Rimantadine, 1100
RING—Resolving infection, 1169
Risk-adapted therapy, lymphoma, 941
Rituximab, 123, 144–146, 787, 789, 810, 838
RNA sequencing (RNA-Seq), 724
Romiplostim (NPlate), 1251, 1253
Rotaviruses, 1092
RSV. *See* Respiratory syncytial virus (RSV)
RSV immune globulin (RSV-IG), 1095
RUNX1 mutation, AML with, 211
Rye classification, 810
Rye–Ann Arbor–Cotswolds staging, 907

S

Salmonella typhimurium, 702
Salvage HCT, 578
Salvage therapy
  chemotherapy, 965
  Hodgkin lymphoma, 918
Sanger sequencing, 56
Sarcoma, myeloid, 219
Satellite dyskeratosis, 1167
SBB stain, 200
Schnitzler syndrome, 624
Screening serologic test, 1108
SCT. *See* Stem cell transplantation (SCT)
Second malignant neoplasms (SMNs), 321
Second-line therapy, for chronic GvHD, 1231
Selective serotonin reuptake inhibitors (SSRIs), 1296, 1297
Senile cardiac/systemic amyloid, 657, 658
Septic transfusion reactions, 1146
Sequence-based typing (SBT), 1196, 1198
  clonal sequencing, 1197
  HLA alleles, 1196
  nucleotide sequence, 1195
Sequence-specific oligonucleotide probe (SSOP), 1194, 1195
Sequence-specific primer (SSP), 1194, 1195
Serotonin norepinephrine reuptake inhibitors (SNRIs), 1296, 1297
Serotonin toxicity, 1297
Serum creatinine, 435
Serum lactic dehydrogenase (LDH) levels, 339
Serum viscosity, Waldenstrom's macroglobulinemia, 626
SES. *See* Socioeconomic status (SES)
Severe allergic reactions/anaphylactic reactions, 1146
Severe combined immunodeficiency syndrome (SCIDS)., 1147
Severe congenital neutropenia (SCN), 469, 1249
Sézary syndrome (SS), 730, 835, 839, 878, 879, 1131
SF3B1 mutation, 106, 493
Short-term liquid storage, of granulocytes, 1168
Siemens Company, Germany, 678
Signal transduction pathways, 486
Simple bag method, 1168
Single-donor histocompatible platelets, 1154
Single-nucleotide polymorphism (SNP), 32, 71, 108, 282, 804, 1197
Single-nucleotide polymorphisms-array (SNP-A) karyotyping, 228
Sinusodial obstruction syndrome (SOS), 1106, 1223, 1224
Sjögren's syndrome, 1018, 1019
Skeletal lesion, osteolytic, 210
Skeletal toxicity, 321
Skin, Waldenstrom's macroglobulinemia, 624
Skin cancer, 766–767
Small bowel obstruction, 925
Small lymphocytic lymphoma (SLL), 79, 81–83, 85, 117, 726, 777, 778, 830
  cytogenetic analysis, 853
  morphology

bone marrow, 83
lymph node, 85
peripheral blood, 81–83
spleen, 85
treatment, 962
Small nuclear RNAs (snRNAs), 171
Small ubiquitin-related modifier (SUMO-1), 414
Smoldering multiple myeloma (SMM)
Bence Jones proteinuria, 534
cytogenetic abnormalities, 534
differential diagnosis, 531, 532
end-organ damage, 531
IMWG criteria, 531
multiparameter flow cytometry, 533
novel imaging assessments, 534
peripheral blood circulating plasma cells, 533
risk of progression, 532–535
serum free light chain ratio, 533
serum M-component evolution, 534
serum M-protein, 532
stratification, 535
stratification and management, 535–536
treatment philosophy, 536
Smudge cell, 82
Social anxiety disorder, 1293
Society of Critical Care Medicine guidelines, 1297
Socioeconomic status (SES), 759
SOCS1 genes, 793
Soft tissue cancers, 675
Soft-tissue masses, 365
Soft-tissue plasmacytomas
assessment, 589
definition, 588
incidence and location, 588–589
plasma cell characteristics, 589
prognosis, 589
treatment, 590
Solid organ transplantation
clinical features, 1013, 1014
epidemiology/demographics, 1012
histopathology, 1013, 1014
immunosuppressive therapy, 1012
type of transplant, 1012
Solitary plasmacytoma of bone (SPB)
adverse prognostic features, 586
clinical findings, 585
diagnostic criteria, 585
multiple, 587
prognosis, 585–586
treatment, 586–587
Somatic events, Waldenstrom's macroglobulinemia, 620
Somatic gene mutation, 106
Somatic genomic. *See* Tumor genomic
Somatic hypermutation, 119
Somatic variant, 363
Sorafenib, 386, 392
South West Oncology Group (SWOG), 535
Spanish Cooperative Group for Hematological Cytogenetics, 863
SPB. *See* Solitary plasmacytoma of bone (SPB)
Spider phobia, 1292
Spleen, 85
Spleen tyrosine kinase (SYK), 81, 246
Splenectomy, 143, 1161
Splenic diffuse red pulp B-cell lymphoma, 787
Splenic marginal zone lymphoma (SMZL), 93, 138, 141, 727, 780, 786, 787, 857
Splenomegaly, 682, 952
Spliceosome, 171–172
Splicing factor 3b subunit 1 (*SF3B1*), 779
Sporadic BL, 796
SS. *See* Sezary syndrome (SS)
Stage IIB bulky disease, 944
Stanford V chemotherapy, 943
Staphylococcus aureus, 1064, 1068
Staphylococcus epidermidis, 1064
STAT3 homodimer, 794–795
STAT5, 32
Stem cell
chronic myeloid leukemia, 36
factor (SCF), 168
maintenance pathway, 37
mobilization, 563
Stem cell transplantation (SCT), 347, 348, 504, 520, 559, 1159
allogeneic, 62, 563
amyloidosis, 662–664
ASCT in, 559
conditioning regimens, 348–349
donor type for allogeneic, 348
eligibility criteria, 559
factors for outcome of, 349
Hodgkin lymphoma, 920
cardiac complications, 921
complications and associations, 925
familial HL, 925
pregnancy and infertility, 924
pulmonary complications, 922
renal disease, 925
second neoplasms, 922
thyroid dysfunction, 925
treatment-related mortality/morbidity, 921
optimal conditioning regimen, 562
stem cell mobilization, 563
tandem transplantation, 561
TTP/HUS, 1275
Waldenstrom's macroglobulinemia, 631
Stereotyped B-cell receptor, 119
Steroid, 345
Storage lesion, 1156
Stored platelets, 1156
Streptococcus mitis, 1080
Streptococcus pneumoniae, 1232
Strongyloides stercoralis, 1058
Subclinical intravascular clotting, 1264
Substance-induced anxiety, 1294
Substance-induced mood disorder, 1295
Subtotal nodal irradiation (STNI), 942
Sulfhydryl groups, 423
SUMO interaction motifs (SIMs), 414, 423
Sumoylation, 419
Sunitinib, 392
Superior vena cava (SVC) syndrome, 308
Supportive care, 375
amyloidosis, 660–661
Supra-frequent donation, 1154
Surveillance Epidemiology and End Results (SEER), 699, 951
Susceptibility, chronic lymphocytic leukemia, 71
Sweet's syndrome, 378
Swiss-type agammaglobulinemia, 702

Symptomatic polycythemia, 1129
Systematic desensitization, 1299
Systemic chemotherapy, 796
Systemic lupus erythematosus (SLE), 1019

T

TAM. *See* Transient abnormal myelopoiesis (TAM)
Tamibarotene, 439, 440
Tamm-Horsfall protein (THP), 602, 603
Tandem transplantation, 561, 577
Targeted therpy, CLL, 94
Targeting antiapoptotic proteins, 124
Tartrate-resistant acid phosphatase (TRAP), 93, 136
TA-TMA, diagnostic criteria, 1225
TAX viral protein, 801
T2Candida, 1071
T-cell
  beta chain antibody expression, 773
  chimeric antigen receptor, 255, 354
  immunophenotype, 308
  lymphomas, 828, 999, 1003
  neoplasms, 776
  NHLs, 773
  progenitor, 298
T-cell acute lymphoblastic leukemia (T-ALL), 203, 297, 298, 799
  with gene expression, 250
  genetic subtypes of, 314
  immunophenotype, 310
  white blood cell count, 351
T-cell epitopes (TCEs), 1201
T-cell large granular lymphocytic leukaemia, 874
T-cell lymphoblastic lymphoma (T-LBL), 799
T-cell non-Hodgkin's lymphomas, 799, 833
T-cell prolymphocytic leukaemia (T-PLL), 873
T-cell receptor (TCR), 255, 297, 777, 827, 830, 1186
T-cell/histiocyte-rich large B-cell lymphoma (TCHRLBCL), 791–792
  cytogenetic analysis, 868
  differential diagnosis from NLPHL, 792
TCF3 mutation, 798
TCF3-PBX1 gene fusion, 310, 314
TD. *See* Thalidomide-dexamethasone (TD)
Technetium DPD, 658
Telomere length (TL), 103
Telomeres, 485
Telomeric fusions, 1026
Terminal deoxynucleotidyl transferase (TdT), 777, 828, 980
Tetracycline transactivator (tTA), 30
T follicular helper (TFH) phenotype, 804
Thalidomide, 664
Thalidomide embryopathy, 520
Thalidomide-dexamethasone (TD), 558
Thaw and wash technique, 1185
Therapeutic and prophylactic platelet transfusion
  acute leukemia, 1157
  chronic hematologic disorders, 1157
  therapeutic procedures, 1159
Therapeutic plasma exchange (TPE), 603
Therapy-induced bleeding and thrombosis
  dasatinib and imatinib, 1274
  ibrutinib, 1274
  L-asparaginase, 1273–1274
Therapy-induced hypoproliferative thrombocytopenia, 1157
Therapy-related acute myelogenous leukemia (t-AML), 465
  clinical presentation, 471–473
  vs. de novo AML, 471
  epidemiology, 466
  epigenetic modulator, 476
  etiology, 466–469
  evaluation, 473
  genetics, 470–471
  hematopoietic stem cell transplant for, 476
  HSC gene signatures, 470
  hypomethylating agents, 476
  ionizing radiation, 468
  karyotypic abnormalities in, 469–470
  non-transplant therapeutic options for, 474–476
  patients with favorable cytogenetics, 474
  P53 mutations, 476
  post-remission therapy, 473
  risk factors, 466
  treatment options, 473
Therapy-related myelodysplastic syndrome (t-MDS), 185, 465, 469
Therapy-related myeloid neoplasm (t-MN), 465, 466
Therapy-related thrombotic microangiopathy, 1275–1276
Thick ascending limb (TAL), 602
6-Thioguanine, 319
Thiopurine *S*-methyltransferase (TPMT), 319
Thioredoxin (Trx), 423
Thioredoxin reductase (TrxR), 423
Thrombin generation, 1263
Thrombocytopenia, 344, 361, 499, 500, 1153, 1157, 1158, 1253, 1256
Thrombocytopenic hemorrhage, 381
Thrombocytopenic leukemia, 1157
Thrombocytosis *See* Plateletpheresis
Thromboembolic events (TEE), 558
Thrombogenic stimulus, 1275
Thrombolytic therapy, 1268
Thrombopoietin receptor agonist (TPO), 499, 1251
Thromboprophylaxis, 600
Thrombotic microangiopathy (TM), 1275, 1276
Thymic alymphoplasia, 702
Thyroid dysfunction, Hodgkin lymphoma, 925
Thyroid hormone RE (TRE), 411
Thyrosine kinase inhibitor (TKI), 203
Tipifarnib, 392
Tissue factor (TF), 441
Tissue factor microparticles (TFMP), 442
Tissue typing, unrelated donor HCT, 1198
TKI. *See* Tyrosine kinase inhibitor (TKI)
T-lineage ALL, 266, 341
T-lymphoblastic lymphoma (T-LBL), 799, 800, 987
T lymphocyte, 702, 703, 708, 714, 715
TMD. *See* Transient myeloproliferative disorder (TMD)
Toll-like receptor (TLR), 107, 618
Toll-like receptor 9 (TLR9), 81
Tongue enlargement, amyloidosis, 653
Topoisomerase 2-beta (TOP2B), 426
Topoisomerase II inhibitors, 467
Topoisomerase II-DNA complex, 467
Total body irradiation (TBI), 1179, 1216
Total therapy, 520
Toxicity, 125–126
  ACP-196 (Acalabrutinib), 127
  BGB-3111, 127
  GS-4059 (ONO-4059), 127
  ibrutinib resistance, 126
  idelalisib, 127–128
  IPI-145 (Duvelisib), 128
  PI3K inhibitor, 127
  second-generation BTK inhibitor, 126
  TGR-1202, 129

Toxoplasmosis, 1057
TP53 gene, 99
  abnormality, 102
  deletion and mutation, 102
  mutation, 102, 791
TP73 gene transcript, 432
Tracheobronchial amyloidosis, 656
Transbronchial biopsy, 1084
Transfusion-associated AIDS, 1147
Transfusion-associated circulatory overload, 1146
  iron overload, 1146
  transfusion reactions, 1146
  transfusion-transmitted diseases, 1147
Transfusion-associated graft-versus-host disease (TA-GvHD), 1141, 1143, 1147, 1148, 1166
  alloimmunization, 1148
  gamma irradiation, 1141
  immunomodulation, 1148
Transfusion-associated hepatitis B, 1147
Transfusion-associated immunomodulation, 1148
Transcription factor (TF), 163–165
  fusion, 162–163
    core binding factor rearrangement, 164–165
    X/RARA, 163
  myeloid, 168
Transfusion-induced alloimmunization (TRAP Study), 1158
Transfusion-induced immunosuppression, 1148
Transfusion medicine, 1039
Transfusion reactions, 1170
Transfusion-related acute lung injury (TRALI), 1146, 1154, 1159, 1166
Transfusion-transmitted human immunodeficiency virus (HIV), 1145
Transfusion-transmitted viral disease, 1155
Transfusion support, transplant patient, 1218–1219
Transient abnormal myelopoiesis (TAM), 289, 368, 369
Transient leukemia, 368
Transient myeloproliferative disorder (TMD), 289, 368
Transjugular intrahepatic portosystemic shunts (TIPS), 1224
Transmembrane domain (TM), 1249
Transmission microscope, 678
Transplant-associated thrombotic microangiopathy (TA-TMA), 1224–1225
Transplantation complication, by transplant type, 1186
Transplant recipients, 1212
Treatment-related mortality (TRM), 476, 1186, 1203, 1211
Tretinoin, 368
Trial to Reduce Alloimmunization to Platelets (TRAP), 1040, 1148
Tricyclic antidepressants, 1297
Trilineage dysplasia, 472
Triple hit lymphomas, 791
Trisomy 12, 102
Trx system enzyme thioredoxin reductase (TrxR), 424
Tumor
  clone, 646
  germline variants and, 74
Tumor genomic, 723–725, 731
  Burkitt lymphoma, 729–730
  chronic lymphocytic leukemia, 726
  clinical practice implication, 741
  diffuse large B-cell lymphoma, 725
  follicular lymphoma, 725–726
  Hodgkin lymphoma, 729
  lymphoplasmacytic lymphoma, 727–728
  mantle cell lymphoma, 726
  MZL, 726–727
  PCNSL, 728–729
  PTCL, 730–731
  small lymphocytic lymphoma, 726
  Waldenström's macroglobulinemia, 727–728
Tumor lysis syndrome (TLS), 53, 979
Tumor-suppressor gene, 35, 102, 119
Twin study, familial predisposition, 731
Typhlitis, 379
Typical CLL, 83
Tyrosine kinase domain (TKD), 167, 245, 383
Tyrosine kinase inhibitor (TKI), 4, 29, 49, 51, 1216
  for pregnancy, 62
  initial therapy of chronic phase, 60–61
  monitoring responses to treatment with, 55–56
  penetrance rate, 13
  resistance, 38
  therapy, 12, 13
T-zone variant, 804

U

Ublituximab, 123
Ultraviolet exposure, non-Hodgkin lymphoma, 766
Umbilical cord blood (UCB), 1180, 1183
University of Chicago Medical Center, 923
Unmutated CLL, 81
Untranslated region (UTR), 1203
Urate nephropathy, 381
Urethane distraction, 519
Urticaria, 1165
US Food and Drug Administration (FDA), 1250
Utilizing ultraviolet B (UVB) irradiation, 1164

V

Valacyclovir, 1081
Valvular heart disease, 921
Vancomycin-resistant enterococci (VRE), 1064
Vanishing bile duct syndrome, 905
Varicella (chickenpox), 1088
Varicella-zoster immunoglobulin (VZIG), 1056
Varicella-zoster virus (VZV), 1056
  diagnosis, 1089
  immunization, 1089
  infection patterns, 1088, 1089
  prophylaxis, 1089, 1090
  treatment, 1089
Vascular endothelial growth factor (VEGF), 609
  plasma and serum levels, 612
VCD. *See* Bortezomib-cyclophosphamide-dexamethasone (VCD)
VD. *See* Bortezomib-dexamethasone (VD)
Vector of incompatibility, 1199
Vemurafenib, 146
Venetoclax, 6, 124
Venlafaxine, 1297
Veno-occlusive disease (VOD), 1106, 1223
Venous thrombosis, 1268
Verax Platelet PGD test, 1166
Vidarabine, 1089
Vinca alkaloids, Hodgkin lymphoma, 696
Vincristine, 696
Viral capsid antigen (VCA), 768
Viral infections, hematological malignancies
  adenoviruses
    infection patterns, 1093
    prevention, 1094
    treatment, 1093, 1094
  CMV, 1082
    diagnosis, 1084
    infection patterns, 1082–1084
    prophylaxis, 1084–1088
    treatment, 1084–1088

Viral infections, hematological malignancies (*cont.*)
cytomegalovirus
diagnosis, 1084–1088
treatment, 1084
EBV
diagnosis and treatment, 1090–1092
pathogenesis and immunity, 1090
hepatitis viruses, 1103
hepatitis A, 1103
hepatitis B, 1103–1105
hepatitis C, 1105–1107
hepatitis G, 1108
herpesviruses family, 1079
HHV types, 1092
HSV
diagnosis, 1080–1082
infection patterns, 1079, 1080
prophylaxis, 1080–1082
treatment, 1080–1082
influenza A and B viruses, 1099
detection, 1099, 1100
prevention, 1101
treatment, 1100, 1101
intestinal viruses, 1092, 1093
papovaviruses (polyomaviruses), 1108
parvoviruses, 1108
PIVs, 1101, 1102
respiratory viruses, 1094
retroviruses, 1108, 1109
RSV, 1094, 1095
diagnosis, 1095
prevention, 1098, 1099
treatment, 1095–1098
VZV
diagnosis and treatment, 1089
immunization and prophylaxis, 1089, 1090
infection patterns, 1088, 1089
West Nile virus, 1109
Viremia, 1083
Vitamin D3 RE (VDRE), 411
Vitamin K-dependent clotting factors, 1274
Volasertib, 393, 476
von Willebrand disease (VWD), 1271
Vosaroxin, 394

W

Waldenstrom's macroglobulinemia (WM), 528, 617, 727, 728, 779, 1010, 1018, 1134
autoantibody activity, 622
clinical features, 621
clinical presentation, 620
clone nature, 617
CNS, 625
cold agglutinin activity, 623–624
course and prognosis, 632–633
cryoglobulinemia, 622
CXCR4 WHIM mutation, 619, 620
epidemiology, 617
eye, 625
funduscopy, 620, 626
gastrointestinal tract, 624
high-dose therapy, 631
hyperviscosity syndrome, 621–622
IgM tissue deposition, 624
IgM-related peripheral neuropathy, 622–623
joints, 624
laboratory findings, 621
blood abnormality, 625
CT, 626
immunologic abnormality, 625
lymph node biopsy, 626
marrow finding, 625
MRI, 626
serum viscosity, 626
lung, 624
marrow film, 618
marrow microenvironment, 620–621
MYD88 mutation, 618, 620
pathogenesis, 617–620
physicochemical/immunological properties, 621
prognostic scoring systems, 632
renal system, 624
response criteria, 631, 632
skin, 624
somatic events, 620
stem cell transplantation, 631
treatment
CD20-directed antibody therapy, 628
combination therapies, 628–630
initiation, 626
maintenance therapy, 630–631
novel therapeutics, 630
nucleoside analogue therapy, 627
oral alkylating agents, 626–627
proteasome inhibitor, 628
Waldeyer's ring, 953
Walter Reed Army Hospital, 702
Washed red blood cells, 1140
West Nile virus, 1109
White blood cell (WBC), 375, 1066, 1129, 1131, 1139, 1140, 1153
common/Pre-B-ALL, 351
immunophenotype, 351
mature B-ALL, 351
Ph/BCR–ABL-positive ALL, 351–352
pro-B-ALL/t(4,11), 351
T-ALL, 351
Whole-exome sequencing (WES), 99, 724–726
Whole-genome sequencing (WGS), 99, 162, 505, 724–726
Wiskott-Aldrich syndrome, 711, 1021, 1026
Withdrawal syndrome, 62
WM. *See* Waldenstrom's macroglobulinemia (WM)
Working Formulation, 704
World Marrow Donor Association (WMDA), 1198

X

X/RARA, transcription factor fusion, 163
X-ray therapy, Hodgkin's disease, 690, 691

Y

Yersinia enterocolitica, 1146

Z

Zanamivir, 1100, 1101
ZAP-70 expression, 120
Zeta-chain-associated protein (ZAP-70), 830
Zoldronic acid, 444
Zygomycetes, 1072–1074